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THE FUNCTION OF THE TRACHEAL GILLS IN LARVAE OF THE CADDIS FLY, MACRONEMA ZEBRATUM HAGEN

(One plate and four figures)

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INTRODUCTION

IT IS generally agreed that water insects arose from terrestrial ancestors and that their aquatic habits and structures were secondarily acquired. Prominent among such structures are tracheal gills, rectal (or anal) gills, and the thinness of the integument that is sometimes characteristic of aquatic insects. Modifications like these are found in caddis-fly larvae, some of which live in damp places but many more of them actually in fresh water of all sorts. Tracheal gills are lacking in larvae of several families; they are variously distributed on the bodies of others, singly or in clusters. The integument of their bodies is generally thin. In the case-building forms the covering of the abdomen is very delicate, and even in some free-living species it is so thin that the finer tracheae are visible through it. In common with other aquatic insects the Trichoptera have a land ancestry and their tracheal gills appear to be secondary structures superimposed upon an aerial respiratory system.

The object of this study was to determine the function of the tracheal gills in larvae of the caddis fly[1] *Macronema zebratum* and

[1] Thanks are due to Dr. Cornelius Betten, of Cornell University, for verifying the identification of the larva.

to determine their relative importance in respiration as compared with the integument of the body. Particular points of inquiry were the closed or open character of the larval tracheal system; the effect of tracheal-gill removal upon the life and respiratory processes of the larva; the effect of their removal upon oxygen consumption and carbon-dioxide elimination; and the efficiency of the tracheal gills in overcoming adverse environmental conditions brought about by reducing the oxygen content of the water or by increasing its carbon-dioxide content. The structure of the rectal gills was examined with reference to their possible respiratory function.

HISTORY

For a long time, the tracheal gills of caddis-fly larvae have been recognized as respiratory organs. In 1734, Réaumur described the gills of *Phryganea* larvae, likening them to those of fishes. Dufour (1847) made the same comparison with the gills of a hydropsychid larva, stating that its external gills were special adaptations for water life, since most insects breathe through spiracles. Larvae of several species of caddis flies were examined by Houghton (1868), who believed that the "membraneous filaments" were organs of respiration. While studying the biology of Trichoptera, Sleight (1913) concluded that certain larvae and pupae breathed by tracheal gills; that others, as thoroughly water-dwellers, breathed through the integument. This was in line with Vayssière's (1882) observations that young May-fly nymphs breathe through the skin, having no gills in their earliest stages. In general the possibilities of the integument in aquatic-insect respiration have been ignored or granted very slight importance compared with tracheal gills.

The rectal gills of certain trichopterous larvae were early described by Pictet (1834), who stated that he found tracheae in them. Müller (1888) described rectal gills in larvae of *Macronema*, believing them to be blood gills but not tracheal gills. The rectal gills of several species of the trichopterous families Rhyacophilidae and Hydropsychidae were studied by Thienemann (1903), who classed those of the hydropsychid larvae as "blood gills" because he found no tracheae in them, and those of *Glossosoma*, a rhyacophilid genus,

as "tracheal-blood gills." Branch (1922) could discover no tracheae in rectal gills of *Hydropsychodes* and termed them "blood gills."

No records of tracheal-gill removal in caddis larvae have been discovered, but this has often been done in other aquatic insects, in some cases as studies in regeneration. Dewitz (1890) took the gills from young May-fly nymphs which lived, molted, and eventually regenerated their gills. In a study of their function Cuénot (1925) removed the gills from nymphs of the May flies *Ephemera vulgata* and *Cloëon dipterum* and concluded that, though not essential to life, they did function in respiration, especially during activity. After making similar experiments on nymphs of *Cloëon dipterum*, Remy (1925) ascribed more respiratory importance to them than Cuénot had given. Dewitz (1890) and Janda (1910) both found that the caudal gills of damsel-fly nymphs could be removed with little ill effect.

A few studies attempt to analyze the respiration of aquatic insects or to determine the function of tracheal gills more definitely. Pruthi (1927) investigated the influence of certain physical and chemical conditions on nymphs of *Cloëon dipterum*, dealing mainly with the effects of carbon dioxide; Remy (1925) also used the method of chemical analysis. The oxygen consumption of normal and operated May-fly nymphs, with all gills removed, was briefly reported by Morgan and Porter (1929).

MATERIAL AND METHODS

Larvae of *Macronema zebratum* live on the under sides of stones in rapid, shallow streams. The larva surrounds itself with a wall of pebbles usually built between stones or in a crevice. The bright-green abdomens make the larvae easy to find. By late fall and winter they are nearly full size, a half-inch or more long. In creeks about South Hadley, Massachusetts, they are very abundant; several may be picked from a stone 4 or 5 inches across. They have conspicuous silvery tracheal gills along the abdomen (Plate I, Figs. 1, 2) and five rectal gills (Plate I, Figs. 6, 7).

Larvae were collected on November 14 and 26 and on December 12. A stock supply of normal individuals was kept in fine wire cages set in constantly running water and supplied with stones and débris

PLATE I

Figures 1, 2, 3, 5, 6, and 9 from living specimens; Figures 7 and 8 from alcoholic ones.

FIG. 1.—Larva of *Macronema zebratum*, showing the tracheal gills, and the rectal ones expanded at the end of the body (length of larva, 15 mm.).

FIG. 2.—Section of the abdomen and tracheal gills.

FIG. 3.—One tracheal gill with its central stalk and filaments.

FIG. 4.—Tips of filaments.

FIG. 5.—Detail of one filament showing a trachea within it. Branches of this bend toward the wall of the filament and course along its inner surface. As it appears under high magnification.

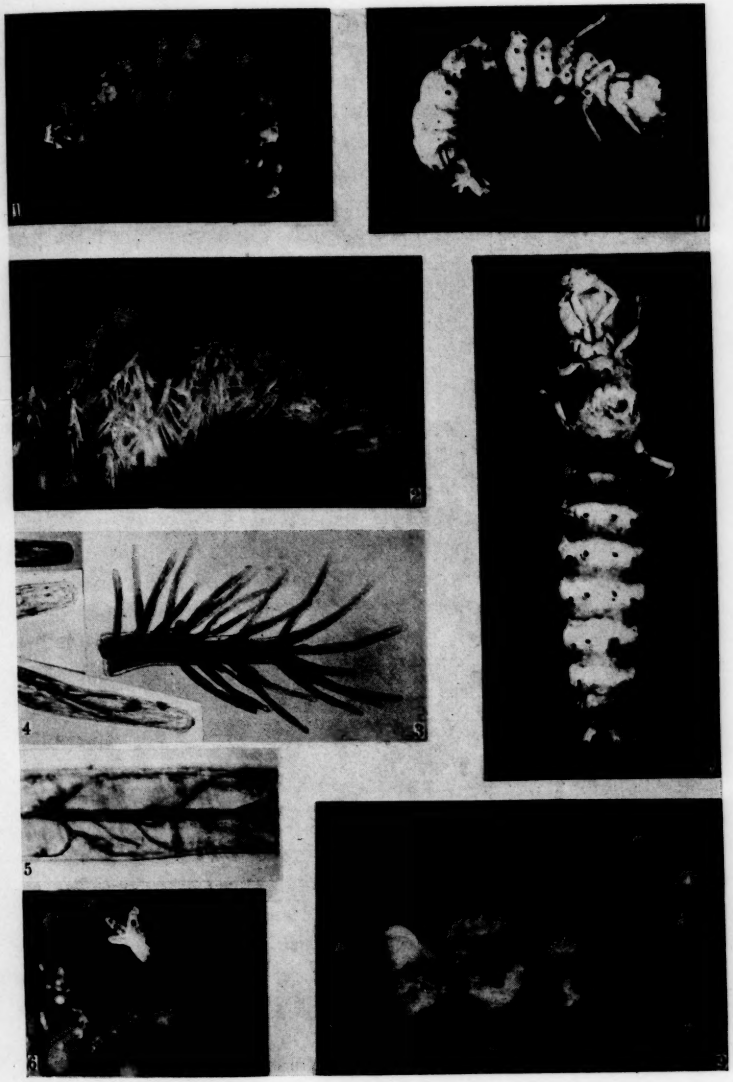
FIG. 6.—Posterior end of the larva with the rectal gills fully distended. Only four gills are visible; the fifth was bent in such a way that it did not show in the photograph.

FIG. 7.—Appearance of the larva 5 months after removal of the tracheal gills.

FIG. 8.—Appearance of the larva 5 months after removal of the tracheal gills. Ventral view.

FIG. 9.—Section of the abdomen showing the gill stubs and the black crust characteristic of the healed surfaces. The thin integument of the ventral body wall appears white.

PLATE I



from their native creek. Larvae from which all tracheal gills had been removed, termed "*gill-less* larvae" in this paper, were also supplied with running water, its temperature varying between 6.5° and 9.5° C. These were kept in laboratory sinks where observation was convenient. Each group of gill-less larvae was placed in a separate glass finger bowl, covered with cheesecloth held in place by a rubber band. Six bowls were supplied with running water from one faucet, the water being first piped into a large bottle from which it was carried to the individual dishes by glass siphon tubes each tipped with rubber. Alga-covered stones were provided for these experimental groups as for the normal larvae.

POSITION AND GENERAL STRUCTURE OF THE GILLS

Gills of *Macronema zebratum* larvae are of two types, tracheal and tracheal-blood (rectal) gills. The filamentous tracheal gills are located on the ventral sides of the mesothorax and metathorax and on the ventral sides of and lateral aspects of the first seven abdominal segments, that is, on all but the last two segments (Fig. 1; Plate I, Figs. 1, 2). Each tracheal gill is made up of a central stalk from which the filaments arise (Plate I, Fig. 3). The lumen of the central stalk contains a bundle of tracheae which branch out into the filaments, there breaking up into finer tracheae that course along the inner surfaces of the filament walls (Plate I, Figs. 3, 4, 5). In the lumen at the base of the living gill-filament blood cells can be seen in the plasma.

The five rectal or tracheal-blood gills are just anterior to the anus on the dorsal side of the ninth abdominal segment (Plate I, Figs. 6, 7). Though no histological sections were made, gross examinations showed that these gills were undoubtedly extensions of the haemocoele. When contracted, they lie within the rectum; but when fully extended, each one measures almost 1 mm. and is distended with the light-green blood. Tracheae enter the lumen of each gill at its base and break up into small branches. Such tracheae are very distinct, and there is no danger of confusing them with muscle, as had been the case in the hydropsychid larva studied by Branch (1922).

TYPE OF THE LARVAL TRACHEAL SYSTEM

The larval tracheal system of *Macronema zebratum* appears to be of the closed or apneustic type in which no functional spiracles are present. No microscopic sections have been made, but careful examination with a binocular microscope revealed no spiracles either on the thorax or the abdomen, a condition usual in gilled, trichopterous larvae.

Simple experiments were made on normal larvae, similar to those made by Portier (1911). Larvae were immersed for a half-hour in olive oil, and in paraffin oil, and then "dried off" on blotting paper. When returned to water, such larvae seemed entirely unaffected by the treatment. Apparently it had not been possible for oil to enter the spiracles, as it had done in the case of Portier's spanworm larva. Larvae were also dropped into a bottle partly full of water, and the air above it was rarefied by means of the faucet aspirator. The pressure was thus so reduced that the air was actually "pulled out" of the tracheal tubes of the larvae. It always escaped from all regions of the body, in greatest amount wherever the integument was thinnest, not localized where spiracles might occur.

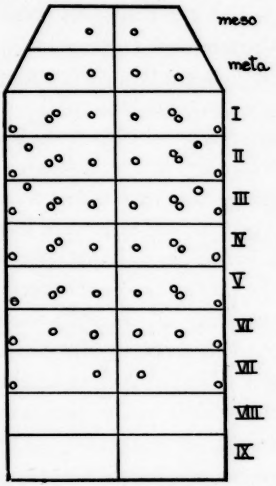


FIG. 1.—Diagram showing the arrangement of tracheal gills on the ventral side of the thorax and abdomen of *Macronema zebratum* larva. *Meso.* = mesothorax; *meta.* = metathorax; I–IX, sections of the abdomen.

The same results were obtained when similar tests were made upon larvae from which gills had been removed 2 or 3 months before. Such larvae were particularly good for observation because the body surfaces (Plate I, Figs. 8, 9) were easier to scrutinize than those of the normal larvae.

All of the evidence thus far attained has pointed to a physiologically closed tracheal system in this larva. Welch (1922) pointed out that Portier's conclusion that the lepidopterous larva, *Nymphula*

stratiotata, had a closed tracheal system could not be proved "without critical morphological work." The same is true of this larva. Although from a physiological standpoint the tracheal system certainly appears closed, only a histological study will give conclusive proof.

REMOVAL OF THE TRACHEAL GILLS

The tracheal gills were removed from well-grown larvae in November and December, 3–4 months before the experiments dealing with respiration were made. During the operation the larva lay in a depression hollowed out in a paraffin bed; here it was held by obliquely set pins crossed over its head and tail regions. If the larva was very active, a weak anesthetic of 2 drops of saturated solution chloretone in 6 cc. of water was added to the water that covered it. The tracheal gills were then removed with iridectomy scissors, under a binocular microscope. In the trial operations it was found that the larvae recovered very soon from the effects of gill removal, and therefore all the gills were cut off at one operation. Operating was made difficult by the delicacy of the abdominal wall and the necessity of cutting close to the root of the gill (Plate I, Fig. 9). Larvae that received the slightest injury were discarded, only 90 operated animals were chosen from the total of 300 larvae on which operations were performed.

During the present investigation gill-less larvae lived 6, 7, and 8 months after gill removal. During that time they built their larval shelters and many of them finally pupated in mid-July of the following summer. Gill-less larvae built shelters and within these they were almost as active as normal ones. When placed upon any fairly smooth surface, however, the gill-less larvae were much more helpless than the others.

The stumps of amputated tracheal gills never showed any regeneration. Black crusts appeared on the cut surfaces about a week after an operation; these appeared to be signs that healing was completed (Plate I, Figs. 8, 9). When operated larvae still incompletely healed were put in water whose pressure was reduced, bubbles escaped rapidly from the stubs. But when those with completely blackened tracheal stubs were treated the same way, there was no such escape of bubbles.

It was very evident that life and at least some of its normal activities were possible to larvae devoid of tracheal gills. It seemed likely that in these larvae the main volume of oxygen intake and the elimination of carbon dioxide took place through the body integument; that probably little of it occurred in the rectal gills, which were kept contracted as much in the gill-less as in the normal larvae.

The part played by the tracheal gills in respiration, and to some extent that taken by the integument and rectal gills, was more clearly defined in the experiments which followed on normal and gill-less larvae.

EXPERIMENTS TO DETERMINE THE FUNCTION OF THE TRACHEAL GILLS

SERIES I. EXPERIMENTS TO DETERMINE THE DIFFERENCE IN OXYGEN INTAKE BETWEEN NORMAL, GILLED LARVAE AND OPERATED, GILL-LESS LARVAE

This set of tests was made to determine the difference in oxygen intake between normal and gill-less larvae. The oxygen determinations were made by the Winkler method.[1] Each of three bottles was filled with 100 cc. of water. In the first one, five gill-less larvae were placed; in the second, five normal larvae whose total weight equaled that of the gill-less ones; the third bottle was left empty being carried as a blank control. A hole was made in each cork, and a tightly fitting glass-tube forced far enough through it so that the tube would project well into the water contained in the bottle. At the other end of the glass tube a piece of rubber tubing was attached; this was clamped by a pinchcock. A half-inch layer of paraffin oil was then poured onto the water within each bottle to prevent the entrance or escape of oxygen from it. The corks were sealed over with paraffin. Samples of water from each bottle were taken at the beginning of the experiment, at the fifth, tenth, and eleventh hours whenever the experiment was continued as long as that. In preliminary experiments some air got into the water when the samples were taken, but with practice this was finally prevented. Titrations were made within an hour after a sampling, and the oxygen con-

[1] Theriault, *U.S. Public Health Bulletin No. 151;* Birge and Juday, *Wisconsin Geological and Natural History Survey Bulletin*, Vol. XXII.

sumption calculated in cubic centimeters per liter. Constant temperature was maintained during the experiment by means of a water bath.

The results of seven experiments are given in Table I. Each experiment consisted of determinations like those given in the record of a typical experiment (Table I, Exp. 6) shown below.

MATERIALS FOR THE EXPERIMENT

Bottle No. 1. Control-blank water sample (determinations made as for Nos. 2, 3)
Bottle No. 2. 5 normal larvae. Weight, 294 mg.
Bottle No. 3. 5 gill-less larvae. Weight, 294 mg.

OXYGEN DETERMINATION AT THE BEGINNING OF THE EXPERIMENT

Bottle No. 1. 7.85 cc. of oxygen per liter
Bottle No. 2. 7.85 cc. of oxygen per liter
Bottle No. 3. 7.85 cc. of oxygen per liter

OXYGEN DETERMINATION AT THE FIFTH HOUR

Bottle No. 1. 7.85 cc. of oxygen per liter
Bottle No. 2. 7.23 cc. of oxygen per liter
Bottle No. 3. 7.08 cc. of oxygen per liter

OXYGEN DETERMINATION AT THE TENTH HOUR

Bottle No. 1. 7.85 cc. of oxygen per liter
Bottle No. 2. 7.08 cc. of oxygen per liter
Bottle No. 3. 6.85 cc. of oxygen per liter

OXYGEN DETERMINATION AT THE ELEVENTH HOUR

Bottle No. 1. 7.85 cc. of oxygen per liter
Bottle No. 2. 6.85 cc. of oxygen per liter
Bottle No. 3. 6.85 cc. of oxygen per liter

Data in Table I show that after the fifth hour of an experiment the oxygen consumption of all the larvae was reduced, whether they were with or without gills. But as an experiment continued, particularly toward the end of it, the oxygen consumption of the gill-less larvae tended to be greatly reduced; and in four experiments that involved twenty animals it ceased altogether (Table I, Expts. 3, 4, 5, 6). This gradual decrease and final cessation in oxygen consumption might have been due to a lowering of the oxygen content of the

water to a minimum beyond which the animals were not able to absorb it, or to an accumulation of carbon dioxide that was toxic to the animals. It is quite possible that both factors operated.

The total consumption of oxygen per liter was the same for gill-less and normal larvae in two experiments (twenty larvae), but in

TABLE I

DETERMINATION OF OXYGEN CONSUMPTION IN NORMAL AND GILL-LESS LARVAE, DURING A PERIOD OF 5 HOURS OR MORE

Experiment	Larvae, Normal or Operated, i.e., without Gills	Number of Larvae in Each Experiment	Total Weight in Milligrams	Temperature of Water Bath in Centigrade	Total Cubic Centimeters of Oxygen per Liter at Start	Total Cubic Centimeters o Oxygen per Liter at End	Cubic Centimeters of Oxygen per Liter Consumed in First 5 Hours	Cubic Centimeters of Oxygen per Liter Consumed in Second 5 Hours	Cubic Centimeters of Oxygen per Liter Consumed in Eleventh Hour	Total Cubic Centimeters of Oxygen per Liter Consumed during Entire Experiment
No. 1.....	Nor.	5	289	7°	8.76	7.99	0.77	.....	.....	0.77
	Op.	5	290	.....	8.55	7.88	0.67	.....	.....	0.67
No. 2.....	Nor.	5	275	7°	8.18	7.80	0.38	.....	.....	0.38
	Op.	5	276	.....	8.18	7.99	0.19	.....	.....	0.19
No. 3.....	Nor.	5	280	6.5°	7.99	7.47	0.38	0.14	.....	0.52
	Op.	5	280	.....	7.80	7.43	0.37	0.00	.....	0.37
No. 4.....	Nor.	5	288	7°	8.18	6.47	1.06	0.27	0.38	1.71
	Op.	5	288	.....	8.18	6.85	0.95	0.38	0.00	1.33
No. 5.....	Nor.	5	291	7°	7.99	7.04	0.87	0.08	.....	0.95
	Op.	5	291	.....	7.99	7.04	0.95	0.00	.....	0.95
No. 6.....	Nor.	5	294	6.5° −7°	7.85	6.85	0.62	0.15	0.23	1.00
	Op.	5	294	.....	7.85	6.85	0.77	0.23	0.00	1.00
No. 7.....	Nor.	10	537	7°	7.85	6.47	1.38	.....	.....	1.38
	Op.	10	536	.....	7.85	6.85	1.00	.....	.....	1.00

the others the normals consumed more (Table I, "Total cubic centimeters of Oxygen per Liter Consumed," Expts. 5, 6). There was no significant difference between the average total oxygen consumption of the normal and gill-less larvae. The total results of the experiments showed that the gills were not essential to oxygen consumption but only accessory and a means of augmenting the oxygen intake.

SERIES 2. EXPERIMENTS WITH NORMAL LARVAE TO DETERMINE WHETHER DECREASED OXYGEN CONSUMPTION IS DUE TO LOW OXYGEN CONTENT OF THE WATER, USING WATER WITH OXYGEN CONTENT AS LOW OR LOWER THAN THAT OF WATER USED IN THE PREVIOUS EXPERIMENT

Experiments were made on normal larvae to find out whether decreasing oxygen consumptions, like those recorded in Table I, were due to low oxygen content of the water or to some other cause.

The oxygen content of the water was lowered by means of a faucet aspirator connected with a glass tube bent at a right angle and run through the stopper of the flask. The water and the larvae to be used were placed in a bottle later tightly stoppered. Five tests were made, each one involving five larvae. The records of a typical one are shown in Table II.

TABLE II

DETERMINATION OF THE OXYGEN CONSUMPTION OF NORMAL LARVAE IN WATER OF LOW OXYGEN CONTENT

Number of Normal Larvae	Weight in Milligrams	Temperature of Water Bath in Centigrade	Total Oxygen in Cubic Centimeters per Liter Present at Start	Total Oxygen in Cubic Centimeters per Liter Consumed	Total Oxygen in Cubic Centimeters per Liter Consumed First 5 Hours	Total Oxygen in Cubic Centimeters per Liter Consumed Last 3 Hours	Total Oxygen in Cubic Centimeters per Liter at End
5	291	7°	5.37	0.57	0.50	0.07	4.80

In the experiment recorded in Table II the oxygen content per liter (5.37 cc.) was lower at the start than the smallest amount (Expts. 4, 7; 6.47 cc. per liter) found at the end of any experiment recorded in Table I. Yet the larvae were able to consume 0.50 cc. per liter of oxygen during the first 5 hours (Table II). This and results of the four other tests indicated that the decreases in oxygen consumption that appear toward the ends of experiments in Table I were not due simply to low oxygen content of the water.

It seemed probable that accumulating carbon dioxide might have had as much, or more, to do with the decrease in oxygen consumption as did the lack of oxygen.

SERIES 3. EXPERIMENTS TO DETERMINE THE EFFECT OF CARBON DIOXIDE UPON NORMAL AND GILL-LESS LARVAE AND TO OBSERVE THEIR POWERS OF RECOVERY FROM IT

Two normal (Figs. 2, 3, 4, Nos. 1, 2) and two gill-less larvae (Figs. 2, 3, 4, Nos. 3, 4) were placed in 100 cc. of water through which carbon dioxide was slowly bubbled (about 100 bubbles per

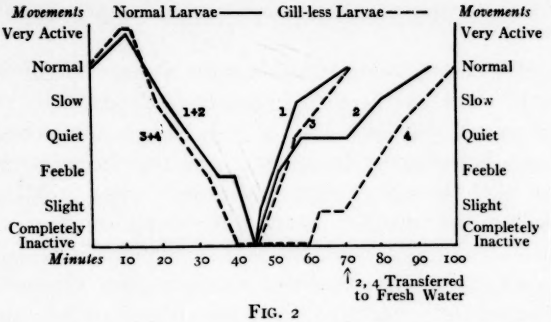


FIG. 2

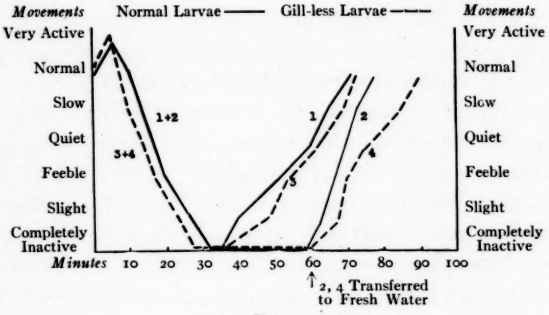


FIG. 3

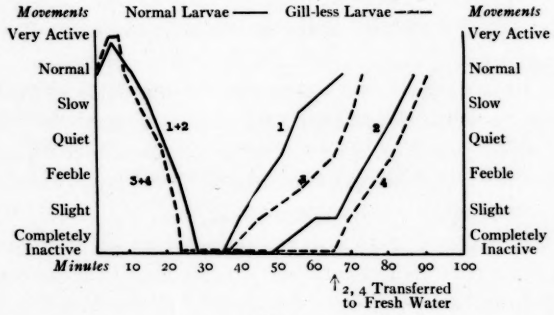


FIG. 4

FIGS. 2–4.—The effects of carbon dioxide on groups of larvae each consisting of two normals (1, 2) and two gill-less larvae (3, 4). Continuous lines record the behavior of the normal, and dotted lines that of the gill-less, larvae.

Lines at the left indicate the increasing inactivity coincident with the increase of carbon dioxide in the water. Lines at the right show the recovery in the water to which the larvae were transferred: 1 and 3 were transferred soon after they became completely inactive, 2 and 4 after an interval as shown. In Figure 2 the water to which larvae were transferred was of normal oxygen content; in the others the oxygen was reduced (Fig. 3, 3.4 cc. oxygen per liter; Fig. 4, 3.02 cc. oxygen per liter).

minute). Observations were made on the movements of these four larvae until they became entirely inactive. Then two of them, one with and one without gills (Figs. 2, 3, 4, Nos. 1, 3) were transferred into an equal amount of fresh water and their recovery observed. The records of three such experiments are shown in Figures 2, 3, and 4. In the first test (Fig. 2) the water to which these two larvae were transferred was of ordinary oxygen content; in the other two (Figs. 3 and 4) the oxygen content was reduced to almost the same level as that of the water from which the larvae had just been taken. The two remaining larvae, one normal and one gill-less (Figs. 2, 3, 4, Nos. 2, 4) were left in the carbon-dioxide-saturated water for an interval of several minutes, following the period of their complete inactivity; then they were transferred to fresh water whose oxygen content was like that used for the other two animals (Nos. 1, 3).

Within a short period (30–40 minutes) accumulating carbon dioxide caused increasing and finally complete inactivity in both normal and gill-less larvae. This result occurred in all experiments of this series and is shown in all three graphs. In each experiment gill-less larvae became completely inactive from 3 to 5 minutes sooner than did the normal ones (Figs. 2, 3, 4, Nos. 3 and 4, 1 and 2). That the gill-less larvae succumbed to inactivity more quickly and maintained the condition longer than normal ones showed that they were more sensitive to carbon dioxide and eliminated it more slowly than the normal larvae.

Both normal and gill-less larvae recover normal movement when transferred to water of ordinary carbon-dioxide content (Figs. 2, 3, 4, Nos. 1 and 2, 3 and 4). The higher the oxygen content the quicker was the recovery. Complete recovery occurred in 25 minutes with an oxygen content of 7.93 cc. per liter (Fig. 2, Nos. 1, 3), in 35 minutes with an oxygen content of 3.4 cc. per liter (Fig. 3, Nos. 1, 3), and in 35 minutes with one of 3.02 cc. per liter (Fig. 4, Nos. 1, 3). Recovery from carbon dioxide was usually slower in gill-less larvae than in normals (Figs. 2, 3, 4, Nos. 3 and 4, 1 and 2); it was sometimes simultaneous (Fig. 2, Nos. 1, 3). Results of these and ten similar experiments indicate that gill-less larvae are not only more sensitive to excess carbon dioxide than normal ones but also recover more slowly from it.

Larvae retained for an interval in the carbon-dioxide water also recovered normal movement when transferred to water of ordinary carbon-dioxide content (Figs. 2, 3, 4, Nos. 2 and 4). In some cases both normal and gill-less larvae made a spurt of activity following the period of complete inactivity. During this recovery, some individuals maintained certain levels of activity for an appreciable time. Figures 2 and 4 show types of this behavior. In other cases both normal and gill-less larvae were still, from the time they became completely inactive until their transference into the fresh water (Fig. 3, Nos. 2, 4).

SERIES 4. TESTS MADE TO COMPARE THE RATE OF CARBON-DIOXIDE ELIMINATION IN NORMAL AND GILL-LESS LARVAE

These experiments were done as further tests, to determine whether normal larvae could eliminate carbon dioxide more rapidly than gill-less ones. The tests were made by Fox's[1] (1921) chemical indicator method. Three vials, each 25 mm. long and 8 mm. in diameter, were filled with haematoxylin indicator solution. A gill-less larva was placed in one vial, a normal one of the same size in another; the third vial was left empty. The corks of all were sealed with melted paraffin. Color changes in the indicator solution were then carefully observed, and the time required for total "bleaching" recorded. Several tests were made, of which the following two are typical.

Test 1. Normal larva; weight, 58 mg. "Bleached" indicator solution in 20 seconds.
Gill-less larva; weight, 58 mg. "Bleached" indicator solution in 1 minute.

Test 2. Stronger indicator solution used, requiring more carbon dioxide for "bleaching."
Normal larva; weight, 55 mg. "Bleached" indicator solution in 15 minutes, 5 seconds.
Gill-less larva; weight, 55 mg. "Bleached" indicator solution in 28 minutes, 46 seconds.

[1] To a dilute solution of aqueous haematoxylin was added just enough potassium hydroxide to change the color of the solution from pink to bluish pink. As carbon dioxide is eliminated from an animal immersed in this non-toxic solution, the color changes from bluish pink to orange and finally to yellow. When the solution turned a very pale yellow it was considered "bleached." This reaction is very clear cut and striking.

In these tests normal larvae were able to bleach the indicator two and three times faster than the gill-less larvae.

SERIES 5. FURTHER TESTS COMPARING THE CARBON-DIOXIDE ELIMINATION IN NORMAL AND GILL-LESS LARVAE. CARBON-DIOXIDE ELIMINATION IN THE RECTAL GILLS

In tests similar to those of Series 4 a normal and a gill-less larva were fastened to paraffin, ventral side up as for operating (p. 368), and the indicator solution poured over them. They were then examined through a binocular microscope. Color changes of the indicator solution were most intense about the abdomen wherever the integument was thinnest. They were more rapid and conspicuous in normal than in gill-less larvae.

Color changes indicated that no more carbon dioxide was eliminated from the rectal or tracheal-blood gills (Plate I, Fig. 6) than through a comparable area of the thin integument of the abdomen. The rectal gills of operated larvae were not extended any more, and no greater carbon dioxide elimination in them was indicated, than in normal larvae in which the tracheal gills were functioning. In none of these larvae was there any evidence of the alternation of function between tracheal gills and rectal gills that has been reported in larvae of a Brazilian *Macronema* (Müller, 1888). Larvae of *Macromena zebratum* give no evidence of using the rectal gills in place of the tracheal gills.

DISCUSSION

The larvae of *Macronema zebratum* not only lived without tracheal gills (except as the rectal ones may be so considered) for eight months, but they built their larval shelters, fastening the pebbles together with silk, and at the end of the time pupated in cases. So long as it was supplied with running water in imitation of its natural habitat, the life of the larva was not interfered with by the loss of its fifty-eight or more tracheal gills, amputated at one time. Tests that were applied to the larvae indicated that the tracheal system was physiologically closed; that is, if spiracles were present, they did not function. Such being the case, the gill-less larvae had no specialized structures left that could act as respiratory organs except the five small rectal gills. In the absence of tracheal gills, they evidently depended upon the thin body integument. Either they sud-

denly and successfully reverted to this thin integument for the exchange of gases between tracheae, blood, and surrounding water or they had so used it all the time. In either case, this is a testimony of the secondary nature of even an abundant development of tracheal gills.

Tests of oxygen consumption of gill-less and normal larvae showed that the gill-less ones fell appreciably behind the others, only after the first 5 hours. It was also true that both normal and gill-less larvae consumed less oxygen in the later hours of the experiment. Since this might have been due mainly to lowered oxygen supply, the oxygen consumption of normal larvae was tested in water whose oxygen content was less than any involved in the previous experiments. During the first 5 hours of such experiments these larvae consumed more oxygen than some of the other groups had done with higher oxygen content. Such results showed that the low oxygen content was not the only cause of the gradual decrease in oxygen consumption.

Similar decreases in oxygen consumption have been encountered in other forms. Dakin and Dakin (1924) found a continually smaller consumption in plaice eggs after the first hour of the experiment. May-fly nymphs of *Heptagenia* weighing 317 mg. consumed 2.4 cc. per liter of oxygen in 5 hours— while another group weighing 350 mg. consumed only 2.9 cc. per liter of oxygen in 24 hours (Morgan and Porter, 1929). And in some cases this has been clearly shown to be the accompaniment of high carbon-dioxide content in the water. In *Planaria*, a combination of high carbon-dioxide and low oxygen content practically abolished oxygen consumption, though without it the oxygen content was sufficient for normal respiration (Hyman, 1925). The carbon-dioxide factor in decreasing oxygen consumption has been demonstrated by Pruthi (1927) in *Cloëon dipterum*. When nymphs were placed in solution having a high carbon-dioxide content, they left at death more oxygen per liter than did similar nymphs in surroundings with less carbon dioxide.

In experiments involving the exposure of normal and gill-less *Macronema* larvae to excess carbon dioxide, the gill-less larvae consumed less oxygen than the normals, succumbed to inactivity sooner and recovered more slowly. Gill-less larvae were very sensitive to

carbon dioxide, and the experiments involving their exposure to it were the main source of mortality among them, many dying even after they had revived. Under natural conditions larvae must sometimes be surrounded by water whose carbon-dioxide content is unfavorable. Under such circumstances tracheal gills would be most important to their existence. Although tracheal gills in *Macronema zebratum* larvae are only accessory in oxygen consumption, they are important respiratory organs in carbon-dioxide elimination.

SUMMARY

1. From a physiological standpoint the tracheal system of *Macronema zebratum* is a closed one.

2. Its whole equipment of about sixty tracheal gills was removed at one time from the larva without serious results. Tracheal gills did not regenerate.

3. Larvae without any tracheal gills lived in otherwise normal condition for eight months, built larval shelters, and finally pupated in cases.

4. Amputation of tracheal gills reduced oxygen consumption slightly, sometimes not at all. In oxygen consumption the tracheal gills of *Macronema zebratum* are only accessory organs, the oxygen intake occurring through the body integument.

5. Normal larvae eliminate carbon dioxide more rapidly than larvae without gills.

6. In the presence of excess carbon dioxide, larvae without tracheal gills consume less oxygen than normal ones, succumb to inactivity sooner, and recover more slowly. In carbon-dioxide elimination tracheal gills are important respiratory organs.

7. The rectal tracheal-blood gills play no significant part in respiration.

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TOXIC AND ANTAGONISTIC PROPERTIES OF Na, Mg, K, AND Ca IONS ON DURATION OF LIFE OF CAMBARUS CLARKII

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THE effect of various ions and their antagonistic properties in respect to the viability and physiological responses of organisms has been the subject of numerous investigations. A comparatively wide range of organisms has been studied among the invertebrates. Definite toxic and antagonistic effects or both have been demonstrated among the Protozoa by Collett (1921) for *Paramoecium* and *Euplotes*, by Walker (1928) for *Colpidium* and *Glaucoma*, and by Berger (1929) for *Colpidium* and *Spirostomum*. Lillie (1906) has demonstrated the injurious action of most sodium salts on the ciliated epithelium of *Mytilus edulis*, which effect, however, can be counteracted by the antitoxic action of the alkali-earth metals and manganese, and to a lesser degree by that of the heavier metals. Heymans (1924) working with the medusa (*Pelagia noctiluca*), Berger (1929) using the larvae of *Rana temporaria* and *Bufo vulgaris*, and Hess (1930) working on *Planaria dorotocephala* have all shown decided toxic and antagonistic ionic effects.

Relatively few workers have concerned themselves with the crustaceans. Of these, however, two merit especial attention. Ostwald (1905) found that *Gammarus pulex* could survive in a solution containing four cations (Na, K, Ca, Mg) when the latter were present in the same proportions as found in sea water. Berger (1929) likewise obtained similar results with *Daphnia*, *Cyclops*, and *Cypris*. As regards the relative toxicity of the ions employed, both workers give the sequence K > Mg > Ca > Na, and describe various ionic antagonistic relationships. A survey of the literature gave evidence, as far as the writer was aware, that no member of the higher Malacostraca (order *Decapoda*) had been utilized in toxicity experiments. For this reason it seemed worth while to determine the reactions of a

typical decapod in order that a comparison could be made with lower Malacostraca and crustaceans in general. Fresh-water crayfish (*Cambarus clarkii*) were selected and suitable experiments devised to test their resistance to pure and mixed ionic solutions, the results of which constitute the text of the present paper.

Materials and Methods

Shipments of *Cambarus clarkii* were regularly received from a commercial supply house, the specimens having been collected in the vicinity of New Orleans, Louisiana, during the months of February–May, 1929, inclusive. Prior to the testing procedure, the crayfish were kept in tap water for several days and then transferred to distilled water where they remained during the 24 hours immediately preceding their immersion in the various salt solutions. During this period the animals were starved in order to standardize this factor, as it might apply to the subsequent resistance of individuals to the salt solutions used.

The various ionic solutions were made as follows: All solutions were made with distilled water in 20-liter quantities. The salts employed were of C.P. quality (Merck) conforming to "Standards of Murray." All solutions were made up fresh immediately before using. The actual testing procedure was briefly as follows: Ten small battery jars were filled with 600 cc. each of the solution to be tested. Crayfish of approximately the same size were then selected (5 males and 5 females) and immersed, individually, in the jars of solution. Every 8–12 hours the jars were emptied and fresh solutions added. This removed any possibility of a reduction in the oxygen supply below the limit of respiratory regulation, the latter point being known for crayfish through previous work of the writer (1928). The tests were run at room temperature, subject to slight variation; and where several concentrations of a single salt or a combination of salts were tested, the tests were run simultaneously.

All crayfish tested were examined at 1-hour intervals, day and night, until death occurred. During the early hours of the test the movement of the entire animal when touched with a glass rod gave evidence of life. Toward the end of the life-span, however, considerable care was necessary to determine whether the animal was actu-

ally dead or not. In many cases the toxic effects of the solution employed were evident for several hours before death occurred. During this period the individuals were unable to move or to right themselves when placed on their backs. The last signs of life consisted chiefly of feeble movements of the appendages and especially of those forming the mouth parts. When the latter ceased to move upon stimulation, the animal was considered dead.

Toxicity of Pure $MgCl_2$, NaCl, $CaCl_2$, and KCl Solutions

The relative toxic effects of pure solutions of $MgCl_2$, NaCl, $CaCl_2$, and KCl were determined in the following manner: 0.5M solutions were prepared of the sodium, calcium, and potassium chlorides; and

TABLE I

Toxicity of pure salt solutions

Concentration of Solutions (Molar)	Duration of Life (Hours)			
	NaCl	$CaCl_2$	KCl	$MgCl_2$
0.5*	20.0	17.0	2.5	12.6
0.45	36.2	25.2	2.9	13.6
0.4	65.9	25.3	3.0	17.8
0.3	157.3 (7†)	32.4	3.9	24.0
0.2	160.2 (6†)	43.1	4.9	65.0
0.1	(10†)	78.8 (2†)	6.5	99.5
0.05	(10†)	95.2 (1†)	17.2	(10†)
Dist. H_2O	(10†)	(10†)	(10†)	(10†)

* The $MgCl_2$ solutions used were twice the molecular strength of the corresponding NaCl, $CaCl_2$, and KCl solutions indicated below.

† Denotes number living for 7 days when test was discontinued.

M solutions of magnesium chloride. A series of weaker solutions were then prepared having the strengths of 90, 80, 60, 40, 20, and 10 per cent of the 0.5M or M solutions. The strengths of the $MgCl_2$ solutions were made double those of the other salts, since preliminary tests had shown that the toxic effects of equimolecular $MgCl_2$ solutions were not strong enough to produce death except in the higher concentrations. In addition to the salt solutions tested, control groups of 10 crayfish each were run simultaneously and individually in distilled water.

The results obtained using the foregoing pure solutions of metallic ions are represented in Table I. Each result represents the average

of those obtained for 10 crayfish. The data indicate that the various cations exhibit distinct and characteristic toxic effects. The K ion is especially poisonous, being approximately eight and seven times more toxic, respectively, than equimolecular 0.5M NaCl and $CaCl_2$ solutions. The toxicity of $MgCl_2$ is relatively much less pronounced than that of KCl or $CaCl_2$, although more effective in general than that of NaCl. The comparison in this instance must be considered as only suggestive, since the $MgCl_2$ solutions were double the molecular strength of those for the other salts. If we consider the toxicity of $MgCl_2$ to be roughly proportional to the molecular concentrations employed, then $MgCl_2$ will fall between $CaCl_2$ and NaCl. The foregoing data indicate, therefore, that for crayfish the sequence of cation toxicity is K > Ca > Mg > Na.

TABLE II

TOXICITY OF PURE KCl SOLUTIONS

Concentration of KCl Solutions (Molar)	Duration of Life (Hours)	Concentration of KCl Solutions (Molar)	Duration of Life (Hours)
0.1	12.7	0.04	21.8
0.09	12.9	0.02	28.8
0.08	13.4	0.01	57.1 (1*)
0.06	15.2	Dist. H_2O	(10*)

* Denotes number living for 7 days when test was discontinued.

The results as represented in Table I also indicate that for Ca, K, and Mg the toxicity of the weaker molecular concentrations is roughly proportional to concentration. This is especially true for K and Mg. Calcium solutions, however, appear to be less toxic, proportionally, as the lower limit of toxic effectiveness is approached. The toxicity of the weaker Na solutions is of interest since their poisonous effectiveness rapidly decreases out of all proportion to the relative molecular concentrations.

The toxicity of a 0.5M KCl solution was productive of such a rapid lethal effect that it was thought advisable to test solutions of one-fifth the former concentrations. The results of this series are represented in Table II. It will be noted that the toxic effect of 0.1M KCl (Table II) was practically exactly one-fifth that of a 0.5M KCl (Table I) solution, in that the life-span of the crayfish was approximately five times as long. This same correlation holds true

when comparing the results of corresponding percentage solutions of 0.1M and 0.5M KCl. Control animals for all series, which were maintained in distilled water, lived for the entire 7 days at which time the tests were discontinued. Ten crayfish were subsequently found to live for over 2 months in distilled water, the inference being that the species under investigation can live indefinitely in this medium.

Antagonistic Properties of Na, K, Ca, and Mg Ions

1. *Antagonistic effects in pure KCl solutions.*—The effect of small quantities of Na, Ca, and Mg ions, added to pure solutions of KCl, on the life-span of crayfish was determined by the preparation of suitable solutions (see solution Nos. 1–19, Table III). When the toxic effect was rapid, as when using 0.5M KCl, the antagonistic effects of the added ions were most pronounced (see solution Nos. 1–7, Table III). All three cations gave pronounced evidence of antagonistic action whether used singly or in combination. In fact, it would appear that the combination of two ions is no more effective than a single ion.

In addition to the 0.5M solutions tested, the antagonistic actions of the same ions were studied in relation to 0.1M KCl solutions. The results of these tests (see solution Nos. 8–14, Table III) were quite negative as regards the demonstration of distinct antagonistic action. The possibility that the amounts of ions employed in solution Nos. 9–14 may have been inadequate to demonstrate noticeable antagonistic action made it desirable to prepare solutions having two and five times the concentrations of the ions previously employed. The nature of these solutions is represented in solution Nos. 16–19, Table III. Reference to the data will show that this assumption was correct. All combinations of Mg, Na, and Ca ions, in the amounts used, were distinctly antagonistic to the toxic effects of the 0.1M KCl solutions. The combination of Ca and Na ions was especially effective, while Ca appeared to be more effective than Mg when in combination with Na. In both solutions where 0.5M concentrations of ions had been added, the antagonistic effects were more pronounced than where 0.2M concentrations had been employed, although not in direct proportion to the increased number of antagonistic ions. In general it should be noted, however, that greater

proportional antagonistic effects were recorded when the toxicity of a 0.5M KCl solution was in question as compared with the results obtained when using 0.1M KCl.

2. *Antagonistic effects in pure $MgCl_2$ solutions.*—The results of adding small quantities of Na, K, and Ca ions to pure solutions of

TABLE III

ANTAGONISTIC EFFECTS OF IONS TO TOXICITY OF PURE SALT SOLUTIONS

Solution No.	Nature of the Solution	Duration of Life (Hours)
1	100 cc. 0.5M KCl	2.5
2	96 cc. 0.5M KCl+4 cc. 0.5M $MgCl_2$	4.1
3	96 cc. 0.5M KCl+4 cc. 0.5M NaCl	4.0
4	96 cc. 0.5M KCl+4 cc. 0.5M $CaCl_2$	4.2
5	96 cc. 0.5M KCl+2 cc. 0.5M $MgCl_2$+2 cc. 0.5M $CaCl_2$	4.2
6	96 cc. 0.5M KCl+2 cc. 0.5M $MgCl_2$+2 cc. 0.5M NaCl	4.3
7	96 cc. 0.5M KCl+2 cc. 0.5M $CaCl_2$ +2 cc. 0.5M NaCl	4.3
8	100 cc. 0.1M KCl	12.7*
9	96 cc. 0.1M KCl+4 cc. 0.1M $MgCl_2$	11.5
10	96 cc. 0.1M KCl+4 cc. 0.1M NaCl	13.0
11	96 cc. 0.1M KCl+4 cc. 0.1M $CaCl_2$	12.0
12	96 cc. 0.1M KCl+2 cc. 0.1M $MgCl_2$+2 cc. 0.1M $CaCl_2$	10.5
13	96 cc. 0.1M KCl+2 cc. 0.1M $MgCl_2$+2 cc. 0.1M NaCl	13.6
14	96 cc. 0.1M KCl+2 cc. 0.1M $CaCl_2$ +2 cc. 0.1M NaCl	12.5
15	100 cc. 0.1M KCl	13.4*
16	96 cc. 0.1M KCl+2 cc. 0.2M $MgCl_2$+2 cc. 0.2M NaCl	13.2
17	96 cc. 0.1M KCl+2 cc. 0.5M $MgCl_2$+2 cc. 0.5M NaCl	17.0
18	96 cc. 0.1M KCl+2 cc. 0.2M $CaCl_2$ +2 cc. 0.2M NaCl	16.0
19	96 cc. 0.1M KCl+2 cc. 0.5M $CaCl_2$ +2 cc. 0.5M NaCl	22.4
20	100 cc. M $MgCl_2$	12.6
21	96 cc. M $MgCl_2$ +4 cc. 0.5M NaCl	16.0
22	96 cc. M $MgCl_2$ +4 cc. 0.5M KCl	10.8
23	96 cc. M $MgCl_2$ +4 cc. 0.5M $CaCl_2$	16.6
24	96 cc. M $MgCl_2$ +2 cc. 0.5M NaCl +2 cc. 0.5M KCl	12.2
25	96 cc. M $MgCl_2$ +2 cc. 0.5M NaCl +2 cc. 0.5M $CaCl_2$	14.6
26	96 cc. M $MgCl_2$ +2 cc. 0.5M KCl +2 cc. 0.5M $CaCl_2$	15.2

* The difference in the average life-span of these two groups of crayfish was no doubt due to the fact that different stocks of animals were utilized, the one used for solution Nos. 15–19 being more resistant.

M $MgCl_2$ are set forth in Table III, solutions Nos. 20–26. It would appear here that Na and Ca are especially antagonistic, while K appears to be much less effective, if functional at all. Combinations of any two ions also appear to be less antagonistic than when using Na or Ca separately. This may be partly accounted for in solution Nos. 24–26 by the presence of K, which, as indicated by solution No. 22, may even tend to increase toxicity.

3. *Antagonistic effects in pure NaCl solutions.*—Similar solutions

were prepared to ascertain the antagonistic effects of Mg, K, and Ca ions in relation to the toxicity of pure 0.5M NaCl solutions (solution Nos. 27–36, Table IV). The antagonistic effects of the Mg and K ions were slight, if any, when used individually; while the effect of Ca ions appeared to be quite pronounced (solution Nos. 27–30, Table IV). Combinations of any two ions, on the other hand, always gave distinct evidence of antagonism. This was especially true

TABLE IV

ANTAGONISTIC EFFECTS OF IONS TO TOXICITY OF PURE SALT SOLUTIONS

Solution No.	Nature of the Solution	Duration of Life (Hours)
27	100 cc. 0.5M NaCl	20.0*
28	96 cc. 0.5M NaCl +4 cc. 0.5M $MgCl_2$	22.7
29	96 cc. 0.5M NaCl +4 cc. 0.5M KCl	21.3
30	96 cc. 0.5M NaCl +4 cc. 0.5M $CaCl_2$	26.0
31	96 cc. 0.5M NaCl +2 cc. 0.5M $MgCl_2$ +2 cc. 0.5M KCl	24.0
32	96 cc. 0.5M NaCl +2 cc. 0.5M $MgCl_2$ +2 cc. 0.5M $CaCl_2$	25.4
33	96 cc. 0.5M NaCl +2 cc. 0.5M $CaCl_2$ +2 cc. 0.5M KCl	33.8
34	100 cc. 0.5M NaCl	33.6*
35	96 cc. 0.5M NaCl +2 cc. M $CaCl_2$ +2 cc. M KCl	34.2
36	96 cc. 0.5M NaCl +2 cc. 2.5M $CaCl_2$ +2 cc. 2.5M KCl	22.0
37	100 cc. 0.5M $CaCl_2$	13.2*
38	96 cc. 0.5M $CaCl_2$ +4 cc. 0.5M $MgCl_2$	12.2
39	96 cc. 0.5M $CaCl_2$ +4 cc. 0.5M KCl	14.9
40	96 cc. 0.5M $CaCl_2$ +4 cc. 0.5M NaCl	14.0
41	96 cc. 0.5M $CaCl_2$ +2 cc. 0.5M NaCl +2 cc. 0.5M KCl	16.5
42	96 cc. 0.5M $CaCl_2$ +2 cc. 0.5M NaCl +2 cc. 0.5M $MgCl_2$	14.7
43	96 cc. 0.5M $CaCl_2$ +2 cc. 0.5M KCl +2 cc. 0.5M $MgCl_2$	12.5
44	100 cc. 0.5M $CaCl_2$	14.2*
45	96 cc. 0.5M $CaCl_2$ +4 cc. M KCl	13.8
46	96 cc. 0.5M $CaCl_2$ +2 cc. M KCl +2 cc. M NaCl	10.8
47	96 cc. 0.5M $CaCl_2$ +2 cc. M $MgCl_2$ +2 cc. M NaCl	11.2

* The differences in life-span were no doubt due to the use of different stocks of animals. Crayfish of one shipment were used in solution Nos. 27–33 and 37–43, while crayfish of another shipment were used in solution Nos. 34–36 and 44–47.

of the Ca and K combination (solution Nos. 31–33, Table IV). Since this latter combination was productive of such a decided antagonistic effect, the attempt was made to still further increase the concentration of this combination of ions (solution Nos. 34–36, Table IV). In this regard it will be noted, however, that doubling the ionic concentration (solution No. 35) tended to remove or neutralize the antagonistic effects of the weaker concentration (solution No. 33), while increasing the concentration to five times the original amount resulted in not only a neutralization of antagonistic effects

but even to an increase in the toxicity of the solution as a whole (solution No. 36). The optimum antagonistic concentration of this combination of ions probably lies, therefore, somewhere near that as indicated in solution No. 33.

4. *Antagonistic effects in pure $CaCl_2$ solutions.*—Solution Nos. 37–47, Table IV, represent the effects of Na, Mg, and K ions in toxic solutions of 0.5M $CaCl_2$. It is at once evident that the addition of small amounts of KCl and NaCl, individually, result in slight antagonism (solution Nos. 39, 40), while $MgCl_2$ alone appears to be ineffective or even slightly additive as regards toxicity (solution No. 38). Where two ions were added to the $CaCl_2$ solution, the combination of Na and K ions was especially effective (solution No. 41). The attempt was made to increase the antagonistic effect of this combination by doubling the concentration of ions. The results indicated, however (solution No. 46), that the increased concentration not only neutralized all antagonism but actually increased the toxic effect. This was likewise found to be true where the concentrations of KCl (solution No. 45) and of the $MgCl_2$ and NaCl combination (solution No. 47) were doubled.

5. *Effect of artificial blood and sea-water solutions.*—The reaction of the crayfish under investigation to salt solutions based on analyses of sea water and of crayfish blood was thought to be of interest. Although numerous analyses of sea water have been made in the past, relatively few determinations of the salt content of crayfish blood were found available for the preparation of suitable analogous solutions. In fact, the writer is aware of but two such determinations: that of Dohrn made in 1866 and that of Griffith made in 1892. Both of these analyses were made with the blood of the European crayfish *Astacus*. Solutions based on these two analyses were consequently prepared. Table V represents the actual amounts of the various salts utilized in preparing 9 liters of solution having the same ionic concentrations as determined by the blood analyses. The average life-span of 10 crayfish was determined following submersion in these solutions and of other solutions based on analyses of sea water. Dohrn's solution proved to be lethal in about 56 hours, while the crayfish placed in Griffith's solution were found alive and healthy at the end of the 7-day test period. The lethality of Dohrn's solution is

no doubt due to the abnormal K and Ca content as determined by this worker. It may be said, in this regard, that Griffith's analysis probably represents more closely the true ionic condition of *Astacus* blood. Crayfish were likewise found to sustain life when placed in Hoffman's double-Ringer's solution. It seems quite likely that the non-lethality of Griffith's and Hoffman's solutions was due to the comparatively low NaCl content as compared with the other solutions of pure NaCl and various antagonistic ions as utilized in the present work.

TABLE V

DURATION OF LIFE IN SOLUTIONS BASED ON ANALYSES OF SEA WATER AND *Astacus* BLOOD

SOLUTION BASED ON—	9,000 CC. OF SOLUTION CONTAINS—									DURATION OF LIFE (HOURS)
	NaCl (Gm.)	KCl (Gm.)	$MgCl_2$ (Gm.)	$CaCl_2$ (Gm.)	$MgSO_4$ (Gm.)	$NaHCO_3$ (Gm.)	NaBr (Gm.)	H_3BO_3 (Gm.)	$CaSO_4$ (Gm.)	
Dohrn's analysis of *Astacus* blood	73.65	25.50	8.57	42.95	.....	.....	.....	.....	.....	56.2
Griffith's analysis of *Astacus* blood	73.65	5.64	1.12	5.59	.....	.....	.....	.....	.....	(10†)
Hoffman's double-Ringer's solution	115.73	2.68	.....	2.00	.....	1.51	.....	.....	.....	(10†)
McClendon's* artificial sea water	271.71	7.31	22.88	11.69	33.48	2.02	0.79	0.60	.....	29.8
Van't Hoff's solution	212.22	5.96	26.97	8.59	16.12	.....	.....	.....	.....	62.8
Back's analysis of North Sea water	212.22	9.09	24.93	.....	17.91	.....	.....	.....	9.99	58.3

* The traces of Na_2SiO_3, $Na_2Si_4O_9$, H_3PO_4, Al_2Cl_6, NH_3, and $LiNO_3$ of McClendon's solution were omitted here.

† Denotes that all 10 crayfish were living at end of 7 days when test was discontinued.

Three solutions based on previous analyses of sea water were also tested. Of these McClendon's solution is of greatest interest since it probably represents more closely the true ionic concentrations typical of sea water. McClendon's solution contains fourteen different salts, but six of these consist of but very slight amounts or traces and were consequently omitted in the solution prepared here. Back's analysis of North Sea water and Van't Hoff's solution (primarily based on Back's analysis) both represent determinations of only the main salts normally present in sea water. The results of crayfish-testing in these solutions indicate that all are decidedly toxic to fresh-water crayfish. Van't Hoff's solution and Back's solution gave

quite similar results, as might have been suspected due to the similarity of their ionic content. McClendon's solution, however, proved to be approximately twice as lethal.

Discussion

The results of the present paper indicate that certain metallic ions exhibit characteristic toxic effects on *Cambarus clarkii* when in pure solutions of their salts in sufficient concentrations. In general the sequence of cation toxicity is the same as determined for other crustaceans (Ostwald, 1905; Berger, 1929), except that Mg appears to be far less toxic for crayfish than previously stated for *Gammarus*, *Daphnia*, *Cyclops*, or *Cypris*. For the latter species, the sequence of toxicity has been stated as Mg > Ca, while the reverse of this would appear to be true for crayfish. It was also noted that no marked correlation existed between the size or sex of crayfish and the relative resistance to the toxicity of lethal solutions.

In general the results of solutions containing two or more ions gave evidence of ionic antagonism for all the metallic ions in question. Usually, however, the concentration of the basic pure salt solution was of such a magnitude as to render complete neutralization of its toxic effect impossible. In fact, the addition of greater quantities of antagonistic salts frequently resulted in an increase of toxicity. When the basic salt solution was of the concentration present in crayfish blood (Griffith's solution) or even somewhat greater (Hoffman's double-Ringer's solution), it was then possible to completely neutralize the toxic effect through the addition of suitable amounts of antagonistic ions. The present work on crayfish differs decidedly from that done on other crustaceans in that solutions containing ions in the same concentrations as found in sea water proved to be distinctly toxic. The possibility, of course, remains that *Cambarus* may be able to sustain life-phenomena if very gradually adapted over long-time intervals from its natural fresh-water environment to the ionic conditions as found in sea water. The idea is an interesting one and merits further investigation.

The possible effect of chemical impurities and the by-products of metabolism on the results of the present work is of importance to consider at this time. Reference to the purity of the chemicals used

has already been made in this connection. Metabolites could not have attained a sufficient concentration to affect the results, since the writer has at times maintained crayfish in water of low oxygen content for months without harmful effect. The fact that fresh solution was applied every 8–12 hours also prevented the accumulation of CO_2 or other waste products sufficient to affect the life of the individuals. No doubt changes in H-ion concentration occurred; but, since the writer (1928) found that not even respiration was affected when the pH was suddenly reduced from 6.8 to 5.2, it seems very unlikely that the much smaller changes in pH which occurred in the present work could have influenced the results.

The criticism might also well be raised that the lethality of the solutions employed was due primarily to their osmotic pressure. That this could not have been the case will be seen by a consideration of the following facts: If osmotic pressure was the primary toxic factor, then a molar solution of $MgCl_2$, which has theoretically at least three times the osmotic pressure of a 0.5M KCl solution, should have killed the crayfish, certainly, more rapidly than the KCl solution. The data, however (see Table I), show just the reverse, since the 0.5M KCl solution killed the animals approximately five times as quickly as the M $MgCl_2$ solution. Other similar comparisons could be made, all showing, with one exception, that the osmotic pressure could not have been the primary factor concerned. This one exception occurs when comparing the results of $CaCl_2$ and NaCl solutions. Here the $CaCl_2$ solutions had approximately a 50 per cent greater osmotic pressure than was true of the NaCl solutions. The greater lethality of the $CaCl_2$ solutions, as compared with the NaCl solutions, might theoretically be explained on this basis, although the other results, which clearly indicate the relative unimportance of osmotic pressure, make this possibility rather unlikely. The results of the solutions (see Tables III, IV, and V) in which small amounts of antagonistic ions were employed, again speak strongly against the osmotic-pressure factor. In these solutions the addition of small amounts of other ions to the pure salt solution could have changed the osmotic pressure of the solution only slightly, and when changes occurred, the tendency would have been to increase the osmotic pressure. As regards lethality, however, these solutions proved less

toxic to crayfish than solutions containing only one salt and having the same or less osmotic pressure. The results, therefore, are typical of the effects of antagonistic ions as they apply to the life-span of many organisms.

The results as determined for crayfish in the present paper must be considered as only roughly indicative of the relative toxicity and antagonism of the ions under question. Several unavoidable factors were encountered which make this generalization necessary. The most important of these is the fact that crayfish exhibit a certain degree of variation in their resistance to any given concentration of an ion or combination of ions. The averaging of the reactions of 10 individuals for each solution tested tended, however, to counteract the individual differences which were recorded. Such individual differences seemed quite probably due to the fact that certain individuals were able to prolong life for several hours especially during the end stages of toxic action, as compared with others immersed in the same solution. This same degree of variation was experienced in a previous work of the writer (1928), where the process of asphyxiation under low oxygen tensions was found to be especially long and drawn out for a certain percentage of the crayfish tested. In general, however, the results justify the sequence of toxicity as concluded to be true for the four metallic ions investigated, although the exact quantitative effect of the various cations present in the different solutions may be subject to later minor modifications.

The results of the present paper, aside from the information obtained regarding the toxic and antagonistic effects of certain ions, have served to supply data of considerable help in the investigation of the effect of various ions on the respiration of crayfish. This latter work is now in progress, and it is hoped that a preliminary report of the effect on respiration of subtoxic and toxic concentrations of individual ions and combinations of ions may be published in the near future.

Summary and Conclusions

1. The poisonous effects of pure solutions of NaCl, KCl, $MgCl_2$, and $CaCl_2$ on a fresh-water decapod (*Cambarus clarkii*) were determined as follows: 0.1M, 0.5M, and M solutions were prepared,

as well as a series of weaker solutions having the proportionate strengths of 90, 80, 60, 40, 20, and 10 per cent of the initial solutions. The average life-duration of 10 crayfish was then determined following immersion in the various solutions. The sequence of toxic cation effect was thus determined as K > Ca > Mg > Na.

2. The possible antagonistic properties of small quantities of other ions in minimizing the toxicity of pure NaCl, KCl, $MgCl_2$, and $CaCl_2$ solutions were also tested by suitable solutions. In general, definite antagonistic effects were recorded for all ions tested, which varied with the nature of the pure salt solution used and the relative proportions of antagonistic ions added. Reference to the detailed descriptions contained in the present paper is essential, due to the variety of results obtained.

3. The effect of various solutions based on previous analyses of *Astacus* blood and sea water, as applied to the resistance of *Cambarus clarkii*, was determined. Solutions based on Dohrn's analysis of *Astacus* blood and of three analyses of sea water proved lethal. Solutions based on Griffith's analysis of *Astacus* blood and Hoffman's double-Ringer's solution proved non-lethal. It is concluded that solutions containing the metallic ions in the same concentrations as present in the blood are non-toxic, while those having the ionic concentrations of sea water are decidedly lethal.

4. The results are discussed in general, and their bearing on future work involving the effect of various ions on respiration is indicated.

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THE EFFECT OF TEMPERATURE ON VESTIGIAL WING IN DROSOPHILA MELANOGASTER, WITH TEMPERATURE-EFFECTIVE PERIODS[1]

(Five figures)

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INTRODUCTION

CONSIDERABLE work has been done in this laboratory upon the effect of temperature on the bar-eyed series of *Drosophila melanogaster* (Driver, 1926; A. H. Hersh, 1924; R. K. Hersh, 1924; Krafka, 1920; Luce, 1926; Seyster, 1919), and the temperature-effective periods have been determined for some of these mutants (Driver, 1926; Krafka, 1920; Luce, 1926). The data of Roberts (1918) and Nadler (1926) suggested that vestigial wing might prove a satisfactory character for similar studies. Experiments were begun in 1927. The writer's brief abstract which appeared in 1928 did not include a consideration of the so-called "critical temperature" for wing length. It was merely stated that the length of vestigial wing varies directly with the temperature, though not in direct proportion, and that there is a distinct sexual dimorphism which is not consistently in one direction at all temperatures but is dependent upon specific temperatures. Also, there is a distinct sexual dimorphism in the percentage of increase in wing length and in the temperature coefficients. It was further pointed out that this marked sexual dimorphism suggests a differential effect of temperature upon accessory factors on the X-chromosome. Temperature-effective periods for wing length were determined at 27° and 17° C. and found to be approximately proportional to the entire developmental period.

MATERIAL AND METHODS

Since careful control of environmental conditions is especially necessary in a study of this kind, the methods used are described somewhat in detail.

[1] Contributions from the Zoölogical Laboratory of the University of Illinois, No. 402.

The stock was the vestigial-winged mutant of *Drosophila melanogaster* obtained from A. H. Sturtevant in the fall of 1927 and carried in single-pair, brother-and-sister matings for ten generations before starting the preliminary experiments. The stock was kept at 25° C., and all routine work was carried on at that temperature.

All the temperatures used, with the exception of 15° and 25° C., were obtained in electrical incubators. Each incubator was equipped with an electric fan which kept the air in continuous circulation and insured a uniform temperature throughout the incubator, for tests had shown that when the fan was not running there was a decided difference in the temperature of different parts of the incubator. The only attempt to control the humidity was to keep an open vessel of water in each incubator. The maximum temperature variation was ±0.3° C. The 15° and 25° C. temperatures were obtained in the constant-temperature rooms in which the incubators were housed. These rooms were equipped with electric fans and have a maximum temperature variation of ±0.5° C.

The cultures were all carried on in 8-dram vials. Each vial contained a $1\frac{1}{4}$-inch square of Scott's Tissue Toweling, which absorbs excess moisture and provides an ideal place for the pupation of the larvae. The vials were closed with cotton stoppers and sterilized for 20 minutes at 15 pounds pressure in an autoclave. About $\frac{3}{4}$ inch of banana-agar food was poured into each vial. The formula for the food was as follows: 150 cc. of soft water, 150 gm. of well-ripened banana, and 3 gm. of commercial agar. When the food was cool, a small pinch of pulverized Yeast Foam was added. It was then allowed to stand at 25° C. for about 2 days before using. Seven pairs of brothers and sisters between 3 and 6 days of age were allowed to lay eggs over a period of 2 hours in each vial. Twelve hours before running a series of laying periods, the flies were introduced to fresh food, where they were allowed to remain for 6 hours and again transferred to fresh food. In the presence of this fresh fermenting food and in the absence of large quantities of eggs the females were stimulated to lay almost continuously. This method insured, as far as possible, eggs which had not been held back by the females and were, therefore, of approximately the same age. During the egg-laying periods the flies were transferred to new food every 2 hours by gently

shaking from one vial to another. The vials containing the eggs were then transferred immediately to the temperature desired. With this procedure each vial usually contained from 6 to 20 eggs. Since optimum food conditions were found in vials containing from 6 to 20 larvae, those producing less than 6 or more than 20 imagos were discarded except at temperatures above 29.5° C., where the mortality was high. Most of the mortality occurred during the pupal period, and the number of pupae formed was used as an index to the number of larvae present.

In the studies on the effect of temperature on length of wing, the flies were etherized and preserved in 95 per cent alcohol for at least two weeks before measuring. Those used in the studies on the temperature-effective periods were slightly etherized and measured in the living condition. All measurements were made with a binocular equipped with a 25 mm. objective and No. 2 oculars, the right one being equipped with a calibrated micrometer. The living flies were placed on a grooved glass plate, the preserved ones in a grooved paraffin block containing a little alcohol. In either case the flies could be easily manipulated into such a position as to throw the wing into clear focus. The right wing was measured in all cases except where there was a distinct difference in the size of the two wings, which occurred frequently in vestigial, especially at temperatures above 29.5° C. In such cases both wings were measured and the mean length recorded. A few flies had to be discarded due to excessive curling of the wings.

In the determination of the temperature-effective periods, several hundred vials were prepared from the same bunch of bananas. These were subjected to 2-hour laying periods at 25° C., placed at the desired temperature, and then changed to the new temperature at regular intervals. These 8-dram vials were small enough to take on the temperature of the new incubator in a few minutes, yet large enough to provide quite satisfactory cultural conditions under the experimental procedure already described. By starting a large number of vials at the same time and making reciprocal transfers, it was possible to work out the temperature-effective periods for two temperatures at the same time, using eggs from the same parents and food made in one batch from the same bunch of bananas.

Preliminary experiments were run in which the eggs were changed every 8 hours at 27° and every 12 hours at 17° C. After the data had been analyzed and the position of the effective period for wing length had been roughly determined, more detailed experiments were planned, involving transfers every 2 hours at 27° C. and every 4 hours at 17° C. throughout the larval period. In order to simplify the tables and graphs in this paper, the data have been condensed to 8-hour intervals at 27° C. and 12-hour intervals at 17° C., except where it was necessary to retain the original groupings in order to make clearer the critical points.

DATA AND DISCUSSION

Table I shows the mean wing length in millimeters and the standard deviations for males and females raised at eleven constant tem-

TABLE I

EFFECT OF TEMPERATURE ON LENGTH OF VESTIGIAL WING

Temperature (Degrees Centigrade)	Males		Females	
	Mean Length (Mm.)	Standard Deviation	Mean Length (Mm.)	Standard Deviation
15	0.569 ± .008	0.068	0.530 ± .011	0.097
17	0.569 ± .008	0.074	0.549 ± .007	0.073
25	0.623 ± .013	0.089	0.666 ± .016	0.089
27	0.667 ± .006	0.060	0.689 ± .007	0.068
28.5	0.800 ± .007	0.078	0.796 ± .007	0.070
29	0.978 ± .009	0.086	0.875 ± .009	0.100
29.5	1.029 ± .012	0.124	0.897 ± .008	0.078
30	1.523 ± .046	0.180	0.940 ± .013	0.101
30.3	1.468 ± .021	0.214	0.974 ± .015	0.157
30.6	1.611 ± .009	0.097	1.333 ± .027	0.285
31	1.643 ± .010	0.074	1.294 ± .033	0.233

peratures ranging from 15° to 31° C. Each mean was calculated from the measurements of approximately 50 flies. In Figure 1, where the same data have been plotted, it will be noticed that there was a sexual dimorphism at all of the temperatures studied except 28.5° C. and at an unknown temperature between 17° and 25° C. The differences in wing length at 15°, 17°, 25°, and 27° C. were not great; but in three separate experiments these variations were in the same directions as those shown in Figure 1 and consequently were considered to be significant. It is very apparent that there is a critical

temperature for wing length. It was noticed in preliminary experiments that this was close to 30° C. for the males, as a slight variation in the mean temperature gave flies of amazingly different wing lengths. The table and figure show that this critical temperature for the males is between 29.5° and 30.3° C. This is shown not only by the slope of the curve but also by the range of variability which is reflected in the standard deviation and the probable error of the mean.

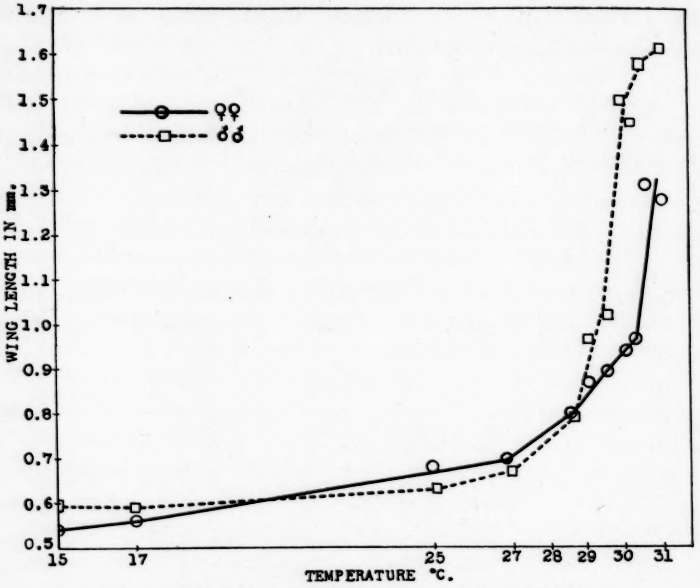

FIG. 1.—Effect of temperature on length of vestigial wing

This variability reached its maximum at 30.3° C. in the males, the range of variation in wing length being from 0.989 to 1.794 mm. The length of the wings increased somewhat up to 31° C., with a marked decrease in variability. It was impossible to raise sufficient numbers for a statistical study above 31° C. under closely controlled conditions. It was found that in a fluctuating temperature (±3.5° C.) the larvae and pupae could stand temperatures as high as 33° C. for short periods. Even at 30.3° C. most of the flies were rendered sterile.

In the females the critical temperature for wing length came between 30.3° and 31° C. The mean wing lengths at 30.6° and 31° C. were much below those of the males at the same temperatures, but the longest-winged female had the same wing length as the longest-winged male (1.794 mm.). At 30.3° C. the actual range of variability in the females (0.713 to 1.587 mm.) was a little greater than in the males, but only a few were long-winged, the majority being grouped at the shorter wing lengths, so that the standard deviation was less than for the males. At 30.6° C. the actual range of variability in the females (1.311 to 1.587 mm.) was much less than at 30.3° C., but the distribution through the classes was more uniform, so that the standard deviation was greater.

As was recently pointed out by Harnly (1930), other marked changes take place in vestigial-winged flies at temperatures above 30° C. An occasional male at 30° C. was found to carry his wings back in the normal long-winged position, and very few carried their wings at right angles to the body as vestigial-winged flies do at 29.5° C. and temperatures below that. At 30.3° and 30.6° C. an increasingly larger number of the males carried their wings in the position characteristic of long-winged flies, while at 31° C. nearly all did so. An occasional female was found at 31° C. to carry her wings in the normal long-winged position, also a single female at 30.6° C. The post-scutellar bristles in a few males at 30.3° and 30.6° C. were directed backward as in the wild type, instead of vertically as is normally the case in vestigial at lower temperatures. Most of the males at 31° C. had the bristles directed backward in the normal wild-type position. A few females at 31° C. and a single one at 30.6° C. also had these bristles directed backward. The width of the wings was also increased at the higher temperatures, but there seemed to be no close correlation between width and length. Some of the longest wings were narrower than many of the shortest ones.

One could not help but be impressed by the close resemblance of these long wings to the figures of Morgan, Bridges, and Sturtevant of the allelomorphic series of vestigial, i.e., strap, antlered, cut, and beaded. Tests are now in progress to determine the possible presence of accessory factors in the stock used.

It has been found that there is not the same correspondence be-

TABLE II

EFFECT OF TEMPERATURE UPON THORACIC LENGTH AND HEAD WIDTH IN VESTIGIAL-WINGED FLIES

Temperature (Degrees Centigrade)	Males		Females	
	Thoracic Length (Mm.)	Standard Deviation	Thoracic Length (Mm.)	Standard Deviation
17	0.861 ± .004	0.037	0.900 ± .004	0.041
27	0.842 ± .003	0.034	0.941 ± .004	0.038
30	0.805 ± .004	0.040	0.892 ± .003	0.032
	Head Width (Mm.)	Standard Deviation	Head Width (Mm.)	Standard Deviation
17	0.837 ± .004	0.037	0.884 ± .003	0.034
27	0.820 ± .002	0.023	0.874 ± .003	0.032
30	0.783 ± .003	0.029	0.835 ± .003	0.028

TABLE III

SHOWING THE MEAN WING LENGTHS OF FLIES STARTING THEIR LIFE-CYCLE AT 27° C. AND COMPLETING IT AT 17° C.

Hours at 27° C.	Males		Females	
	Mean Length (Mm.)	Standard Deviation	Mean Length (Mm.)	Standard Deviation
0	0.577 ± .008	0.058	0.556 ± .009	0.082
4	0.564 ± .008	0.060	0.552 ± .008	0.080
8	0.553 ± .007	0.080	0.574 ± .010	0.091
12	0.558 ± .006	0.073	0.559 ± .008	0.077
16	0.542 ± .008	0.077	0.550 ± .007	0.069
18	0.561 ± .006	0.086	0.553 ± .007	0.073
24	0.559 ± .005	0.076	0.584 ± .004	0.069
32	0.587 ± .006	0.077	0.598 ± .006	0.077
40	0.585 ± .006	0.069	0.621 ± .012	0.076
48	0.607 ± .005	0.079	0.641 ± .005	0.083
56	0.633 ± .004	0.075	0.664 ± .005	0.079
60	0.643 ± .012	0.121	0.674 ± .016	0.138
62	0.668 ± .009	0.077	0.715 ± .008	0.056
64	0.688 ± .004	0.064	0.716 ± .004	0.060
72	0.713 ± .004	0.065	0.721 ± .007	0.075
76	0.698 ± .006	0.056	0.702 ± .005	0.069
88	0.678 ± .008	0.068	0.710 ± .009	0.069
112	0.683 ± .007	0.058	0.691 ± .011	0.077
136	0.658 ± .009	0.063	0.669 ± .012	0.103
160	0.666 ± .007	0.058	0.662 ± .017	0.147
184	0.658 ± .007	0.066	0.678 ± .013	0.106

tween changes in wing length and in body length in vestigial as appear (in unpublished data) in wild-type flies of this species. Table II shows the greatest head width and greatest thoracic length of vestigial-winged flies raised at three temperatures. It is fully appreciated that these measurements are, at best, poor indexes of body size, but they surely indicate that the flies are smaller at 30° C. than at 27° C. The difference in size between 27° and 17° C.

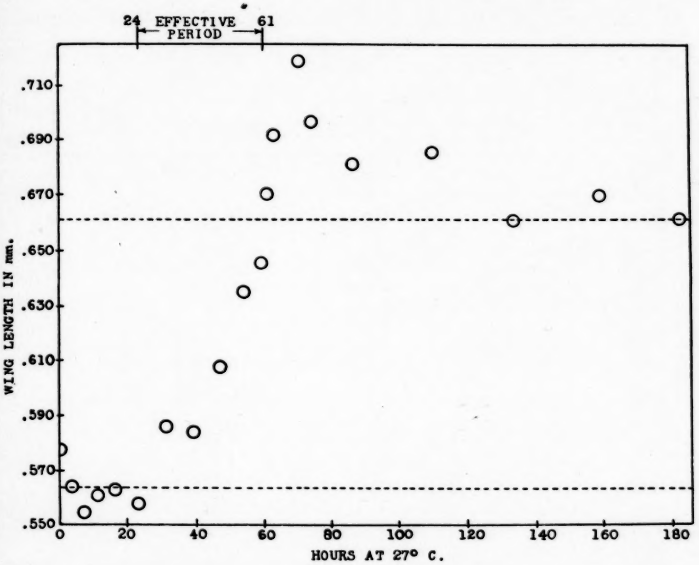

FIG. 2.—Data for the males started at 27° C. and transferred to 17° C. to complete their development.

is not so clear. Comparison of these data with the wing lengths given in Table I make it apparent that the relationship, if any, is inverse.

Table III, Figure 2, and Figure 3 represent the mean wing lengths of vestigial flies beginning their life-cycle at 27° C. and being transferred to 17° C. at the intervals indicated (computed from the middle of the 2-hour egg-laying period to the hour of transfer). The lower dotted line in each figure represents the mean wing length

of 200 flies from the same parents, raised under the same experimental conditions and spending their whole life at 17° C. The upper dotted line in each figure represents a similar group of flies spending their whole life at 27° C. As has been pointed out above, the data have been condensed to 8-hour intervals with the exception of a few groups near the critical points. Each circle, therefore, represents

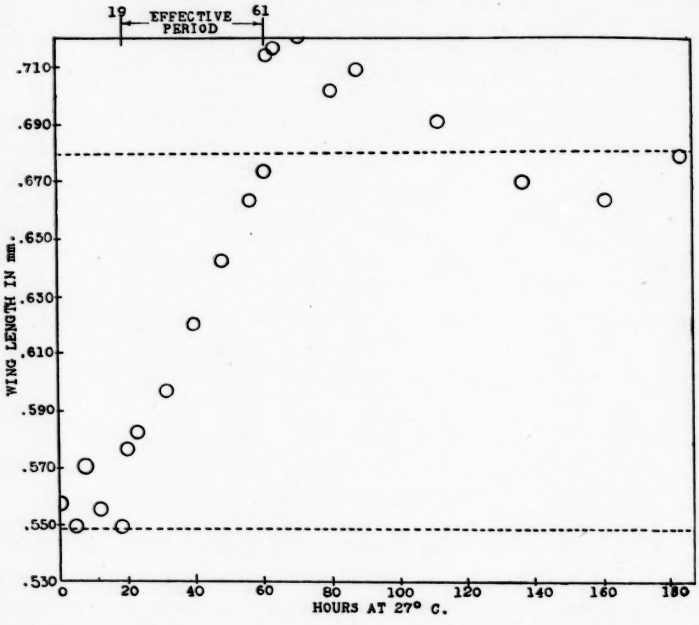

FIG. 3.—Data for the females started at 27° C. and transferred to 17° C. to complete their development.

the mean wing length of a group of flies varying in most cases from 50 to 200 in number.

Just as the temperature-effective period at 27° C. was determined by transferring flies from 27° to 17° C. at the intervals indicated, so the effective period at 17° C. was determined by reciprocal transfers. The results of the transfers from 17° to 27° C. are shown in Table IV and Figures 4 and 5.

The temperature-effective period is interpreted as beginning at the approximate time when the flies deviate significantly from the mean of the temperature to which they were transferred, and to end when they reached the mean wing length of the temperature at which they were started. It will be noticed that there are humps in each of the curves, those of the 17° to 27° C. transfers being less

TABLE IV

SHOWING THE MEAN WING LENGTHS OF FLIES STARTING THEIR LIFE-CYCLE AT 17° C. AND COMPLETING IT AT 27° C.

Hours at 17° C.	Males		Females	
	Mean Length (Mm.)	Standard Deviation	Mean Length (Mm.)	Standard Deviation
0	0.647 ± .008	0.072	0.679 ± .010	0.093
24	0.680 ± .007	0.068	0.720 ± .009	0.078
48	0.653 ± .006	0.048	0.697 ± .008	0.072
72	0.657 ± .006	0.059	0.681 ± .007	0.068
96	0.656 ± .004	0.064	0.687 ± .006	0.076
108	0.660 ± .005	0.058	0.681 ± .005	0.060
120	0.651 ± .006	0.069	0.675 ± .006	0.075
132	0.651 ± .005	0.056	0.656 ± .006	0.066
144	0.640 ± .006	0.058	0.657 ± .006	0.070
156	0.587 ± .015	0.080	0.659 ± .010	0.074
168	0.592 ± .011	0.067	0.615 ± .007	0.100
180	0.600 ± .012	0.088	0.625 ± .006	0.076
192	0.581 ± .008	0.067	0.581 ± .008	0.081
204	0.569 ± .006	0.060	0.554 ± .007	0.074
216	0.552 ± .006	0.064	0.543 ± .006	0.065
228	0.551 ± .007	0.069	0.538 ± .006	0.071
240	0.545 ± .006	0.073	0.531 ± .006	0.071
252	0.540 ± .013	0.080	0.527 ± .006	0.071
264	0.564 ± .008	0.069	0.552 ± .018	0.091
276	0.551 ± .005	0.068	0.555 ± .014	0.096
304	0.562 ± .011	0.085	0.533 ± .006	0.049
328	0.560 ± .016	0.059	0.540 ± .013	0.069
348	0.558 ± .007	0.051	0.548 ± .009	0.069
372	0.544 ± .016	0.058	0.522 ± .007	0.056

conspicuous. That is, for example, the flies transferred from 27° to 17° C., for a few hours after what appears to be the end of the effective period, have longer wings than those spending their entire life at 27° C. The preliminary experiments gave similar results. If the humps in the curves are significant, it might be argued that the effective-period had not yet ended. It would seem that the transfer exerts a stimulating effect. Further study is being made of this phenomenon at several different temperatures.

In the males beginning their life-cycle at 27° C., it appears that the temperature-effective period for wing length begins at about 24 hours after egg-laying and ends at about 61 hours, giving an effective period of approximately 37 hours. In the females at this temperature the period starts at about 19 hours after egg-laying and seems to end at about 61 hours, having a duration of about 42 hours.

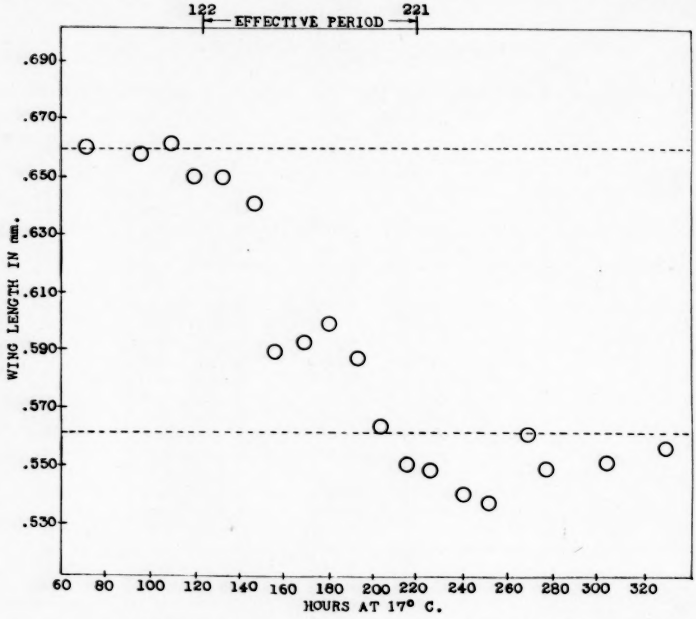

FIG. 4.—Data for the males started at 17° C. and transferred to 27° C. to complete their development.

In the males beginning their life-cycle at 17° C. and being transferred at the time-intervals indicated, it appears that the effective period begins between 120 and 124 hours after egg-laying and ends between 218 and 224 hours; that is, the effective period is approximately 99 hours. The effective period for the females at this temperature seems to begin between 122 and 126 hours and to end between 218 and 224 hours, having a duration of approximately 97 hours.

The average length of larval life, computed from the laying of the eggs until about half the pupae had formed, was 121 hours at 27° C. and 326 hours at 17° C. The average period from egg-laying to emergence of half the imagos was 194 hours at 27° C. and 494 hours at 17° C.

It is impossible to draw close correlations between the present study and the work of Alpatov (1929) on the three instars of wild

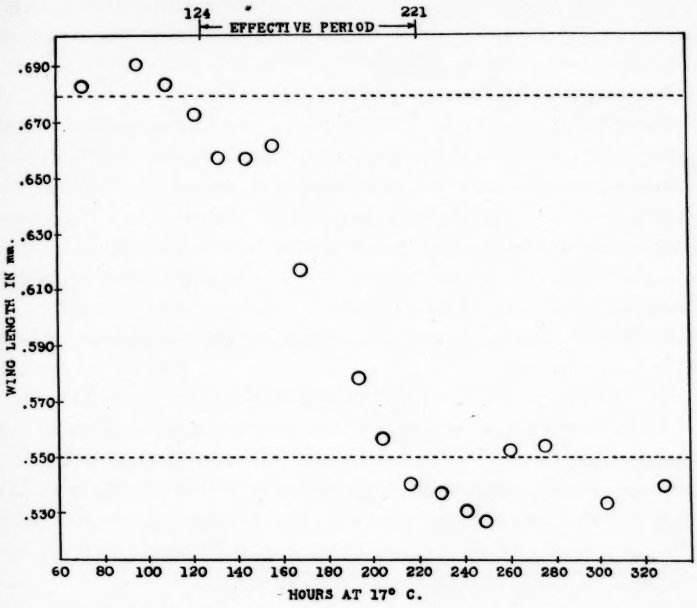

FIG. 5.—Data for the females started at 17° C. and transferred to 27° C. to complete their development.

Drosophila melanogaster raised at 28° C., on account of differences in the temperatures and stocks used and in the methods of computing the time-intervals. However, since development at 28° C. is somewhat more rapid than at 27° C., and since vestigial is considerably slower in development than wild flies, it would seem that the effective period for wing length in vestigial at 27° C. occurs during the first instar and part of the second instar.

Chen (1929) reported that the earliest trace of the dorsal mesothoracic bud was found in larvae 16 hours after hatching (raised at 25° C.), and that the wing bud appeared at the end of larval development. The length of vestigial wing at 25° C. is not much different from that at 27° C., nor is there much difference in the length of the developmental periods. It seems quite clear, then, that the effective period for length of vestigial wing begins at 27° C. before the mesothoracic bud forms and ends at about the time of its appearance (or shortly afterward), which is considerably earlier than the first trace of the wing buds.

The length of the effective period for the males at 27° C. is approximately 37 per cent of that at 17° C., and for the females about 43 per cent. The larval life at 27° C. is approximately 37 per cent of that at 17° C., while the developmental period from the egg to the imago at 27° C. is about 39 per cent of that at 17° C. The effective periods for the two temperatures, therefore, seem to be roughly proportional to the larval periods and to the entire developmental periods from the egg to the imago.

Studies are now in progress to determine the effective periods for other temperatures.

ACKNOWLEDGMENTS

The writer wishes to express his sincere appreciation to Dr. Charles Zeleny, under whose direction this study was carried out, for helpful suggestions and kindly interest while the work was in progress. The writer also wishes to express his thanks to Mr. H. C. Oesterling, editor of the Illinois State Natural History Survey, for assistance in the preparation of the manuscript.

SUMMARY

1. An unselected, inbred strain of the vestigial-winged mutant of *Drosophila melanogaster* has been raised at eleven constant temperatures ranging from 15° to 31° C.

2. The wings of over 7,500 flies, raised under the experimental conditions described in this paper, were measured and recorded and constitute the data of this study.

3. The length of vestigial wing varies directly with the temperature at which the flies are raised but not in exact proportion.

4. There is a distinct sexual dimorphism of wing length at all the temperatures studied except at 28.5° C. and at an unknown temperature between 17° and 25° C.

5. This sexual dimorphism is not consistently in one direction at all temperatures but is dependent upon the specific temperatures.

6. There is a critical temperature for length of vestigial wing.

7. The critical temperature for the males is between 29.5° and 30.3° C., as determined both by extreme changes in wing length and by range of variability.

8. The critical temperature for the females is between 30.3° and 31° C., as determined both by extreme changes in wing length and by range of variability.

9. At these temperatures there are manifested also changes in wing width, in the carriage of the wings, and in the angle of elevation of the post-scutellar bristles.

10. No definite correlation was found between body size and wing length.

11. The temperature-effective period for length of vestigial wing has been determined for 17° and 27° C. by the method of transfers at short intervals.

12. This temperature-effective period occurs during early larval life, apparently falling within the first and second instars.

13. The temperature-effective period at 17° and 27° C. is approximately proportional to the larval period and to the entire developmental period from the laying of the egg to the emergence of the imago.

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STEREOTROPISM AND GEOTROPISM OF THE SALAMANDER, TRITURUS TOROSUS

(Five figures)

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IN GENERAL, a physiological function whose correct regulation is of vital importance to an animal is governed not merely by one mechanism but rather through the co-operation of many—the principle of multiple assurance. Thus, vertebrates maintain their position in space by means of impulses emanating from various sense organs, including labyrinths, eyes, proprioceptors, and end-organs of pressure and touch. The relative importance for orientation of each of these varies greatly among the different types of animals. Maxwell (1923) has shown that the bilaterally labyrinthectomized dogfish, although completely unoriented while swimming freely, upon contact with the wall or bottom of the aquarium will so orient itself that the ventral surface comes in contact with the object touched. The animal's behavior is unchanged by blinding. This demonstrates how stereotropism may aid or even partly replace the labyrinthine functions, and that in the dogfish the eyes are of no importance in this connection. On the other hand, a labyrinthectomized dog, held on its back, will lift its head so as to regain its normal relationship to its surroundings, merely through visual stimuli (Magnus, 1924). If the animal be blindfolded, the head falls backward and hangs limply. Moreover, a labyrinthectomized rabbit, laid on its side, will not right itself if a lightly weighted board be placed upon it, because of equal tactile stimulation of both sides. Again the importance of stereotropism in equilibrial reactions is demonstrated.

Moore (1929) has proved the salamander to be strongly stereotropic. Since stereotropism has been shown to be of such great importance in connection with labyrinthine reflexes in other animals, I thought it of interest to study their relationship in this amphibian. Besides, urodeles are the most primitive animals existent possessing

that bodily structure which, differing greatly from the architecture of fishes, is common among the higher animals, i.e., the tailed quadrupeds. Since the salamander does not possess binocular vision, it could not be expected that visual reflexes would play any important part in its orientation to a static visual field.

The first reference to the function of the labyrinth of a urodele was that of Ewald (1892) on *Salamandra maculosa*. He found, after either unilateral or bilateral labyrinthectomy, a slight disturbance of time-interval in the sequence of leg movements in walking. Moreover, a unilaterally labyrinthectomized animal, while walking, lifted the legs on the normal side very high in the air, while those on the operated side dragged on the ground.

Laudenback (1899) noticed that removal of one labyrinth caused strong rolling movements in the swimming of *Siredon pisciformis*. A specimen lacking both ears was entirely disoriented in water but, upon touching the bottom, righted itself. Steinmann (1914) rotated *Triton* larvae and adults while they were in water, and found that locomotion occurred in the opposite direction. Both nystagmus during rotation and response to a rotating visual field were absent.

Stewart (1922) published his results on the equilibration of *Necturus maculatus*. Animals with but one ear showed typical rolling movements toward the injured side. When both labyrinths were lacking, the salamanders sometimes exhibited rolling movements but more often made strong bending movements from side to side.

By far the most complete account of the labyrinthine reactions of a urodele has been offered by Greene and Laurens (1923) on *Amblystoma punctatum*. These workers extirpated the rudimentary sense organ in early embryos and studied the result throughout development.

EXPERIMENTAL

STEREOTROPISM

In addition to those stereotropic reflexes of *Triturus* as described by Moore (1929), I have observed several others. If, while the animal is being held by the tip of the snout, with the body hanging freely in the air, one rubs the dorsal portion of the tail at the root, the tail bends dorsally. Likewise, stimulation of the ventral surface

of the base of the tail, just posterior to the legs, will produce a strong ventral bending of the tail. This reaction may be observed during the normal life of the animal as it crawls over a rounded object, such as a rock. Due to the rubbing of the ventral surface, the tail bends ventrally and so comes into closer contact with the surface of the object.

As will be explained later, a labyrinthectomized animal held by the tip of its tail does not hold its head horizontal, as does the normal, but allows it to hang straight down. If the belly is rubbed with an instrument, the animal bends itself ventrally (emprosthotonus) in a positive stereotropic reflex (see Fig. 1). The normal animal does not respond to such a stimulus, except for a slight emprosthotonus of the body. The position of the head remains unchanged. In the normal animal the labyrinthine stimuli are apparently stronger than the stereotropic ones; but if the labyrinths are removed, stereotropism prevails. Hence, in normal life these two reflexes are presumably algebraically added.

An interesting example of inhibition may be observed while producing the leg-clasping reflex. Moore (1929) proved that stimulation of the area between the legs causes a strong clasping of the legs about the stimulating object. Usually, in order to demonstrate this phenomenon, the animal is held by the tip of the tail. If, however, the salamander is held by the body, this clasping reflex becomes weak and variable. The strength and certainty of the response is inversely proportional to the amount of body surface in contact with an object, such as the hand. Apparently, stimuli originating from the end-organs of touch in the skin, other than from the area between the legs, tend to inhibit the clasping reflex.

This effect is comparable to inhibition of the knee-jerk reflex in mammalian preparations by a stimulation of the ipsi-lateral sensory nerve of the foot. Even more analogous is the response of the manubrium of a medusa (Bethe, 1903). If the margin of the bell is touched at one point, the manubrium bends toward the source of stimulation. If the margin is now touched on the opposite side, the manubrium relaxes, proving that the inhibition is caused by collision of impulses. Moore (1926) has demonstrated a similar phenomenon in the ctenophore *Beroe*. He states:

Gentle mechanical stimulation on one side of the anterior end of the animal causes homolateral contraction of the longitudinal musculature so that the posterior end is curled upward and forward. If now while continuing the first stimulation weak mechanical excitation is exerted on the side opposite to the first, the "tail" not only straightens but relaxes. Here again impulses of approximately equal strength arrive from opposite directions and annul each other. The result is inhibition of muscular activity.

GEOTROPISM

A salamander held by the tip of its tail and hanging freely in the air assumes an opisthotonic position, with back arched and the head bent backward so that the labyrinths assume practically their normal position with respect to gravity (see Fig. 2). If put on an inclined plane, the animal also tends to keep its head level as far as possible. Due to the fact that the head is carried so low that it barely clears the ground, marked tilting is impossible, although even when the animal is held in various positions in the air, the head exhibits only a slight response. Moreover, no distinct tonus changes in the legs are apparent, and the salamander does not change its direction of locomotion in response to any tilting of the plane, except when the degree of tilting makes it mechanically impossible for it to proceed in that direction. If the head of the animal is held in its normal position in relation to the body, any consequent change in the position of the head relative to gravity seems to produce practically no deviation of the eyes. In fact, the eyes of this animal seem to be incapable of noticeable movement in any direction.

If a salamander on the surface of a table is turned over (on its longitudinal axis) toward its left side, the tail is quickly bent toward the right, as if to counteract the displacing force by its weight. As the animal is forced entirely over on its back, the bent tail will now lie on the table at the salamander's right side, and by its position will tend to prevent any further rolling toward that side. Then, by arching its back, the animal is left balancing on its nose and the base of its tail in a marked opisthotonic state, resulting in an unstable equilibrium. Since the tail lying on the salamander's right side acts as a prop, making it mechanically impossible for it to roll toward that side, it falls toward the opposite side (left) and easily rights itself, in the opposite direction to that toward which it was

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 1.—A doubly labyrinthectomized salamander, while hanging head downward, is rubbed on the ventral surface with a rod. The animal bends ventrally toward the source of stimulation.

FIG. 2.—The normal salamander, while hanging by its tail, tends to maintain its head horizontal by assuming an opisthotonic attitude. Note that the body and tail do not form a straight line, but that the tail is bent somewhat dorsally.

FIG. 3.—The hind feet of a salamander lacking the left ear were dipped in ink. The animal was then allowed to walk over white paper. The blurring of the tracks on the left caused by dragging the foot is easily discernible.

FIG. 4.—A doubly labyrinthectomized animal no longer holds its head horizontal or its limbs in fixed symmetrical positions. Compare with Figure 2.

FIG. 5.—A salamander stimulated by repetitive mechanical stimuli on its back, as by rapping with a rod, exhibits a strong opisthotonus. Notice the retracted and tightly closed eyes and the tonic posture of the legs.

turned. As a result it again rests on the same spot from which it was first moved.

If *Triturus* is placed upon its back with its tail in a line with its body, it may bend the tail to one side and right itself as above, or may regain its normal equilibrium by strongly flexing its head backward and rolling to one side or the other.

When rotated upon a turntable, the animal's head bends in the direction opposite that of rotation but without head nystagmus, and a tonus change becomes apparent in the legs, the ones on the side toward rotation being bent while those opposite are extended. During slow rotation the salamander may begin to walk opposite the direction of rotation along the edge of the confining vessel. If the rotation is fast, it pushes itself to the opposite side by means of its stretched foreleg, so that it turns about in a very small circle, the head approaching the tail. In fact, the circle is often so small that the animal walks over its tail. Sometimes, it seems as if the tail is actively bent, opposite to rotation, bringing it nearer the head, so as to form a ring. Far more often, however, it seems to lie passive; but because of the small circle described by the salamander, the head approaches the end of the tail, making it appear to the casual observer that both ends were actively approaching each other. Upon sudden stopping of the turntable, the head is brought back to the normal position, or slightly past it. Sometimes, the salamander will start crawling in the former direction of rotation if the speed of rotation has been sufficient. Removal of visual stimuli by surrounding the animal with a high opaque cylinder fails to produce any noticeable change in behavior.

In order to determine the extent to which the labyrinths are concerned in the orientation of this form, experiments were carried out on animals in which one or both labyrinths had been destroyed. The operation of destroying the labyrinth was performed in the following way: the salamander was anesthetized with ether, laid on its back, and the mouth opened widely. Through the skin of the roof of the mouth the white otoliths can be seen. A sharp probe is inserted into this cavity and moved about, breaking up the structure of the labyrinth and otoliths. There is neither loss of blood nor detrimental effect upon the animal.

UNILATERAL LABYRINTHECTOMY

Immediately following recovery from the effects of the anesthetic, the head of the animal is markedly tilted toward the operated side.

The eyes show no discernible deviation in any direction.

There are no distinct tonus changes evidenced in the position of the legs, either while on a table or when the animal hangs by its tail. Nevertheless, while walking, the legs on the normal side are lifted higher than those of the opposite side, the latter often dragging on the table top as they move forward in stepping (see Fig. 3). This is probably due to a slight tilting of the body toward the operated side. Neither rolling nor circus movements are present while the animal is in or out of the water. However, if while in water the animal is strongly stimulated, as by rough handling, severe pinching, etc., it may execute rolling movements toward the operated side. These continue until it reaches the bottom or side of the aquarium, when it rights itself.

When a specimen is rotated toward the operated side, there is no reaction, whether visual stimuli are present or excluded. At cessation of rotation there is a movement of the head in the former direction of rotation, or the animal may even crawl in that direction. If the rotation is toward the normal side, the reaction is similar to that of a normal animal, but postrotatory effects are absent.

BILATERAL LABYRINTHECTOMY

A salamander lacking both labyrinths is with difficulty to be distinguished from the normal while at rest either in water or on land. When walking on a smooth surface out of water, however, its head is quite unoriented, and swings slowly from side to side and up and down. Often the forepart of the body is raised so high that the animal's equilibrium becomes unstable and it falls on its side.

The stereotyped righting reflex of the normal animal has completely disappeared. When the labyrinthectomized animal is placed upon its back, only after much struggling does it finally regain its feet, as a result of differential stereotropism. If suspended in the air by its tail, the body and head hang straight downward and the legs assume various asymmetrical positions (see Fig. 4).

During swimming, the animal is completely unoriented, showing

strong rolling movements, circling, etc., but upon touching the bottom of the aquarium, soon rights itself, as a result of stereotropism. The animal is never found lying on back or sides while in contact with the bottom. All responses to rotation are lacking.

OPISTHOTONIC REFLEX

During the course of these experiments I noticed that if a *Triturus* was dropped on the floor from a height of several feet, it assumed an opisthotonic attitude, the head and tail bending backward. Further experimentation showed that repeated dropping caused a summation of effect until such a strong opisthotonus developed that the animal formed a complete ring, the head and end of the tail meeting over the back. The eyes were tightly closed and the legs stiffly extended (see Fig. 5). Tapping the spinal column with a glass rod will produce the same response, which gradually irradiates under repetitive stimuli and slowly departs when the stimuli cease. Until the normal posture has been regained, the salamander seems to be in a "hypnotic" state, similar to that produced in other animals by various well-known means.

Strychnine (0.0002 gm.) injected under the skin so facilitates the process of excitation that very light taps on the vertebrae suffice to produce a strong response. Eviscerated-decapitated animals still react in the same fashion, although the intensity of the response is somewhat diminished.

It seemed to me that this phenomenon could be due only to stimulation of either nerve endings in the skin or the nerve elements in the cord. In order to eliminate the epithelial end-organs, an ethyl-chloride spray was used. The efficacy of this as an anesthetic was proved by spraying for a few seconds a small area between the legs of a normal animal. Rubbing this treated area caused no response; whereas stimulation outside of this area produced the normal clasping reflex of the legs. This proved that complete anesthesia of the sensory elements of the skin could be readily effected in this manner. The spray was then applied to the back and sides of a normal animal, and the back was tapped, only to find that the vigor of the response was unchanged. Hence, I believe that this phenomenon must be due to a direct mechanical stimulation of the motor neurones

of the cord. That the musculature is so widely involved is accounted for by the fact that motor neurones of similar function are connected in linear fashion throughout the cord, as demonstrated by Coghill (1929) and Moore (1929).

I know of no similar phenomenon among other animals. Of course, a nerve may be readily stimulated by a mechanical shock. If then, a peripheral axon is excited mechanically, there should apparently be no reason why either an axon or nerve cell in the central nervous system should be irresponsive to an identical stimulus. Muscular responses have been noted upon stimulation of the cortex or white matter of the brain by various stimuli, including electrical, chemical, and mechanical. Hence, it is to be expected that the same results may be obtained with the spinal cord. The lack of response to blows upon the back of other animals than *Triturus*, such as the frog, may be due to the better mechanical protection of the cord by overlying muscles, heavier vertebrae, etc., or—what is more probable—to a different neurological organization.

Verworn (1896) was able to secure a remotely similar reflex in the decerebrate frog by a mere rubbing of the skin of the back. The animal then strongly arched its back (emprosthotonus) and stiffly extended its legs. Here, however, a skin receptor is involved.

The slow disappearence of the muscular contraction upon cessation of stimulation argues for a prolonged after-discharge of the nerve elements concerned, i.e., a gradual dissemination or catabolism of Sherrington's E-substance.

DISCUSSION

Ewald (1887) showed that, among birds, labyrinthectomy produced greater disturbances in those birds which ordinarily perform more rapid and intricate movements (such as the swallow), as compared to the transitory disturbances exhibited by the goose. These differences he explained as depending upon dissimilitudes in the acuity of the vestibular sense, and on external conditions such as the mechanical stability of the body, the surrounding medium, etc. Pike (1923) correctly assumes that the degree of development of both the exteroceptive (and I would add interoceptive) sense organs and the central nervous system is also essential to explain such non-

agreement. Another element which seems to me of importance is the fact that the labyrinths are very sensitive regulators of movement and posture, compared to their auxiliary aids such as stereotropism. Hence, in a slow-moving labyrinthectomized animal these secondary mechanisms for the maintenance of posture are allowed a greater time in which to function. The rapid movements of other organisms may not be of sufficient duration for the successful functioning of less delicately tuned mechanisms. This may be demonstrated in a unilaterally labyrinthectomized *Triturus*. During slow swimming, the animal behaves quite normally. If stimulated to rapid movement, the remaining labyrinth cannot compensate sufficiently, and the animal continually rolls on its longitudinal axis. Consequently it is not surprising that we find dissimilarities in the effects of labyrinthectomy among the various genera of salamanders. From observations of *Triturus* and *Ambystoma* sp., I know that in comparison *Triturus* is very sluggish in all its movements. It is readily captured by hand, even when swimming; whereas the larva of another salamander (unidentified) living in the same habitat is secured only with the greatest difficulty, owing to the rapidity of its movements. It is to be expected, therefore, that labyrinthectomy of such a slow-reacting salamander as *Triturus* may not produce disturbances equivalent to those of *Ambystoma*.

The results obtained by the various workers on *Siredon pisciformis*, *Triton cristatus*, *T. alpestis*, and *Necturus maculatus* agree in practically all the main points, in so far as the work was carried. In the case of *Ambystoma punctatum* there are many differences, for in this animal stimulation or lack of the labyrinths produces stronger effects than in *Triturus*. Among the most important points of nonagreement between the reactivities of *Ambystoma* and those of other salamanders is the presence of head nystagmus in the former during rotation. *Ambystoma* is therefore the lowest animal in the scale to respond in this manner; whereas head nystagmus is a common phenomenon observed in the rotation of reptiles, birds, and mammals. Corresponding, perhaps, to the better developed response to rotation, *Ambystoma* also presents noticeable changes in the position of the legs upon destruction of one vestibule. This effect, which is lacking in *Triturus*, has been proved, in the frog (De Kleyn, 1914)

and mammals (Magnus, 1924), to be mediated by a neck reflex. Possibly this neck reflex is absent in *Triturus*.

Concerning the general effects of unilateral labyrinthectomy, Maxwell (1923, p. 33) states:

> The destruction of one labyrinth or the section of one eighth nerve gives certain results which seem to be common to all classes of vertebrates. Prominent among these are characteristic forced positions of the eyes and head and of the organs of locomotion. The eyes tend to deviate to the operated side; at the same time the eye on the sound side goes slightly upward. Eye nystagmus is often present for some days, with the slow movement to the operated side. The head is inclined so that the operated side is lower than the sound side. In animals like the fishes where the head is not movable on the neck, the body is also inclined to the operated side. In amphibians, reptiles, and mammals, there is a tendency to extension or adduction of the limbs on the sound side.

To this I would add the fact that the head is usually bent toward the operated side, as in *Ambystoma* and mammals. Nevertheless, removal of one labyrinth produces only a small fraction of the disturbance caused by extirpation of both ears.

The results of the removal of one ear in *Triturus* do not completely agree with the general statement by Maxwell (1923) cited above. The tilting of the head toward the operated side is present, as well as a slight tilting of the body; but the consequent dragging of the feet on the operated side seems to be a unique phenomenon.

The lack of distinct tonus changes in the legs does not agree with results of Greene and Laurens (1923) upon *Ambystoma*, in which case there was also a bending of the head toward the operated side. Both of these effects are lacking in *Triturus*. Moreover, in no salamander has there been noted deviation or nystagmus of the eyes due to the operation. *Triturus* also does not swerve to the injured side, as does *Ambystoma*.

On the whole, the eyes of *Triturus* are of little importance in equilibrial reactions. Since the salamander probably does not possess binocular vision, it could not be expected that the doubly labyrinthectomized animal would right its head when suspended in the air, as do the higher mammals, such as the dog. Likewise, when swimming, a unilaterally labyrinthectomized salamander is unable to orient itself through visual stimuli.

During rotation neither *Ambystoma*, *Triton*, nor *Triturus* show

any marked influence of moving retinal images, as is the case in lizards (Trendelenburg and Kühn, 1908).

Greene and Laurens (1923) have noted, upon unilateral blinding of *Ambystoma*, a tilting of the head toward the normal side. Completely blinded animals often exhibited opisthotonus, especially when excited. They offer no explanation of these phenomena. From previous work on fishes (Main, 1928), which also tilt toward the normal eye when the opposite one is not functional, it seems that the explanation of this response in *Ambystoma* may likewise be due to phototropism. *Triturus* does not exhibit any change in behavior upon either unilateral or bilateral blinding.

The opisthotonic position assumed by *Triturus* when turned on its back, which results in righting, is quite dissimilar to the reactions of most vertebrates. McNally and Tait (1925) state "That the head should preserve its normal orientation for the proper guidance of movements connected with equilibrium is indicated by innumerable cases." In *Triturus*, however, the head is bent downward, rather than upward, as would be the case for most animals. None of the other workers upon urodeles have mentioned their righting mechanisms.

This reflex is shown practically unchanged in animals with but one labyrinth but has totally disappeared in those lacking both. Since the double labyrinthectomized animal does finally right itself after considerable struggling, it must be due to stereotropism, since a blinded one acts likewise.

CONCLUSION

Labyrinthine reflexes in *Triturus* closely resemble those of other salamanders, with the exception of *Ambystoma punctatum*, which exhibits greater responses to vestibular excitation or extirpation. This fact agrees with the results of Ewald (1887), who discovered great differences in the reactions of various types of birds to labyrinthectomy, depending upon their nervous development and other conditions.

In comparison to higher vertebrates, *Triturus* exhibits fewer reactions mediated by the inner ear, and less disturbance upon their removal. (However, we must except from this statement those

vertebrates, which, like the dog, may, by means of visual stimuli or other means, compensate for the loss in function of the end-organs of the eighth nerve.) The explanation of this comparative lack of disturbance may be due to the strong stereotropism of the animal, its mechanically stable equilibrium, and an apparent relative lack of those specialized neuro-muscular mechanisms necessary to many animals for the maintenance of equilibrium, such as are exemplified by various head and neck reflexes.

SUMMARY

Rubbing the base of the tail of *Triturus* results in a bending of the tail toward the stimulus. Similar stimulation of the belly of a labyrinthectomized animal produced a ventral bending, which is inhibited in the normal salamander by labyrinthine excitation. An inhibition of the leg-clasping reflex is produced by stimulation of the touch receptors in the skin other than those which produce the reflex.

Triturus tends to maintain its head in its normal position to gravity. If the animal is turned over on its back, it executes a stereotyped righting reflex, necessitating the assumption of an opisthotonic position. During rotation the head is bent in the opposite direction, together with tonus changes in the body and legs which result in the movement of the animal.

Unilaterally labyrinthectomized animals tilt the head toward the normal side, drag the feet on the operated side due to the tilting of the body, and show rolling movements during swimming if strongly stimulated. They respond, as do the normal ones, when rotated to the uninjured side, but do not respond to rotation toward the operated side.

Bilaterally labyrinthectomized animals no longer orient their head. They also show disturbances in walking and are completely unoriented while swimming.

Repetitive mechanical shocks upon the dorsal side of this salamander produce a remarkable opisthotonus, which may be caused by direct stimulation of the motor neurones of the cord.

I am indebted to Dr. Olof Larsell, Dr. Ernst Gellhorn, and especially to Dr. A. R. Moore, for friendly criticism and advice.

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POLARITY IN NORMAL AND CENTRIFUGED EGGS OF URECHIS CAUPO FISHER AND MacGINITIE

(Six figures)

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INTRODUCTION

DURING the summer of 1929 while testing the eggs of this echiuroid worm for egg-fragment studies, it was found that owing to their exceedingly tough membrane these eggs could not be cut with a quartz micro-needle. It was further noted that the eggs, when shed into the water, are immature and normally do not undergo maturation until after the entrance of the sperm. It was also observed that numerous red granules which, together with other inclusions, are dispersed throughout the cytoplasm of immature eggs, migrate during cleavage along the astral rays and come to encircle the nucleus of each daughter cell.

Following some preliminary experiments, it was decided to investigate this egg by means of the centrifuge in order (1) to test the durability of the egg membrane and viability of the cell as indicated by subsequent development, (2) to determine the degree and nature of stratification of the cell contents, and (3) to ascertain if centrifuging the immature eggs at high speeds over relatively long periods of time might influence the position of formation of polar bodies and the direction of the polar axis with reference to that of the centrifuging axis.

The literature bearing upon similar problems for which the centrifuge method was employed is extensive, and eggs of various groups of animals have been used for this purpose. References to most of this literature will be found in recent general texts such as Wilson's *The Cell in Development and Inheritance* (3d ed., 1925), Cowdry's *General Cytology* (2d ed., 1928), and Morgan's *Experimental Embryology* (1927). Especially the latter text (see chap. xii) gives a critical review of the more important papers. It is not necessary, therefore, to attempt to give here the results of these previous inves-

tigations, although for purposes of comparison and discussion mention should be made of the general conclusions that are shared in common and of certain results which bear more directly upon the work now to be reported.

It seems to be generally agreed that the visible cytoplasmic inclusions of all eggs which have thus far been subjected to strong centrifugal force do not represent formative substances. The induced stratification of these inclusions shows that yolk is ordinarily the heaviest inclusion, since in yolky eggs it is forced to the centrifugal pole, while oil or fatty materials come to lie toward the centripetal pole, thus leaving the hyaline cytoplasm, the nucleus, and any other inclusions to occupy the middle zone. Normal development may follow in eggs thus stratified by centrifuging, and the axis of stratification does not bear any known relation to the axis of polarity of the centrifuged egg.

Since the original axis of polarity is apparently unaltered by centrifuging, it is evident that the organization of the egg, a primary mark of which is polarity, cannot be causally related to any of the inclusions that are stratifiable by the magnitudes of centrifugal force and the periods of its duration thus far applied. Polarity, then, must inhere in some other substance of the egg. Lillie (1906) was the first to identify this substance. In centrifuged eggs of *Chaetopterus*, Lillie showed that their original polarity was maintained in further development irrespective of their axis of stratification or of the induced position of the nucleus. He concluded, therefore, that "polarity is a property of the ground substance of the protoplasm." These results were confirmed in a later study (Lillie, 1909) wherein it was further demonstrated that bilaterality was also unmodified in the centrifuged *Chaetopterus* eggs. His thesis that the hyaline ground substance constitutes the seat of vital organization was re-emphasized, and evidence was presented that would indicate a molecular basis of this primary organization of a persistent, optically homogeneous medium.

A similar view is maintained by Conklin (1910) in his experimental studies on the eggs of fresh-water pulmonates where he found no correspondence between the stratified materials of the centrifuged eggs and their definitive polarity or bilaterality. Accordingly, he

looked to some "tenuous framework which interpenetrated the entire cell and which is not disturbed by the shifting of the visible substances of the egg" as the essential material in which polarity and bilaterality inhere. No direct evidence for this framework could then be offered "distinct from the hyaline protoplasm"; but in a later paper (Conklin, 1917), which presents the results of centrifuging the eggs of *Crepidula*, he comes to identify definitely this "tenuous framework" with a "viscid spongioplasm" which forms a peripheral layer around the egg and a continuous framework throughout the cell that connects the nucleus and the centrosphere or the mitotic figure. This spongioplasm is characterized also by its elasticity and contractility, which may permit extreme stretching of the spindle and its nuclear and peripheral connections, followed by partial or complete recovery after centrifuging. Consequently, the essential relation of parts—of nucleus, centrosphere, mitotic figure, etc.—remained inviolate.

The pioneer work of Lyon (1906, 1907), in centrifuging the eggs of *Arbacia*, showed that the planes of cleavage tended to correspond with the induced stratification of the egg. Lyon suggested that this might denote a modification of the original polarity of the egg. His results in general were confirmed by Morgan and Lyon (1907), although his suggestion regarding a modification of polarity or bilaterality was apparently not valid.

The latter workers showed that gastrulation occurred at any point on the stratified egg of *Arbacia*. In the majority of cases, however, the micromeres were formed at the centrifugal or pigmented pole and the archenteron appeared on one side of the pigmented pole. Moreover, in the majority of plutei the pigment lay between the mouth and anus to one side of the median line. But since this pigment might be found over other parts of other plutei, it was concluded that there was "no necessary relation between the stratification of the materials as such and the embryonic axes."

Similar conclusions were reached by Morgan and Spooner (1909) from studies also on centrifuged *Arbacia* eggs. Although the early cleavage planes corresponded with the axis of stratification, the micromeres tended to appear directly opposite to the micropyle but always at the intersection of any two of the first three planes of

cleavage. Deviations in this diametric relation of the micropyle and micromeres might, therefore, occur; but subsequent regulation of a "labile stereometrical arrangement of the molecules" of the ground substance effected normal development.

In centrifuged eggs of *Ascaris*, Boveri (1910) found some deviation from normal development when the applied force was continued until the egg completed or nearly completed its first cleavage. Both of the resulting blastomeres gave rise to germ cells and soma cells up to the 4-cell stage, whereas in normal development one of the first two blastomeres (that one toward the animal pole) is entirely somatic. Boveri accounted for this anomalous behavior in the centrifuged egg from the fact that its plane of first cleavage is polar and not equatorial, as in normal *Ascaris* eggs. Each blastomere of the centrifuged egg, therefore, received equally material of the animal hemisphere, which material normally determines the diminution of the chromosomes in the first soma cell.

It should be noted that in some of the eggs used in the foregoing experiments maturation had begun (*Chaetopterus*, *Ascaris*) or was completed (*Arbacia*) before the centrifugal force was applied. Gasteropod eggs (*Lymnaea*, *Physa*, *Crepidula*), however, were centrifuged by Conklin (1910, 1917) while in their germinal vesicle stage, as well as during and after maturation and fertilization and in various stages of cleavage. It is well known that these various stages may not be comparable in the eggs of different species, as has been clearly demonstrated by egg-fragment studies (Wilson, 1929), although the degree of development is, of course, indicated by such stages in eggs of a given species. But whether centrifuged in an earlier or a later period of development, no marked differences in the results were obtained by Conklin for eggs in either of the three gasteropod species noted. In view of the results about to be reported, however, it is of interest to refer to his Experiment 4 (1910, p. 427) on eggs of *Lymnaea*, in the germinal vesicle stage, which were centrifuged at 600 gravities for 20 minutes. He notes that "the separation of substances was very sharp" and that the first maturation spindle, which usually lay oblique to the axis of stratification, appeared near the boundary between the clear and gray zones where the first polar body was extruded about 1 hour after the centrifuging.

The second polar body was given off, as usual, near the first. Of 25 eggs, "all but 5 had the polar bodies on the clear zone" (p. 428). Their cleavages were normal in form, although the distribution of the stratified materials was abnormal. It should be stated further, however, that considerable irregularity in the position of one or both polar bodies was observed by Conklin (1917) in centrifuged eggs of *Crepidula*, which led him to conclude that the variable induced position of these polar bodies did not necessarily coincide with the original axis of polarity.

MATERIAL AND METHOD

The echiuroid worm *Urechis caupo* was recently discovered in abundance in the mud-flats of Elkhorn Slough adjoining Monterey Bay about 20 miles from the Hopkins Marine Station. In addition to the two papers (Fisher and MacGinitie, 1928*a*, 1928*b*) describing this species, further investigations (unpublished) by the latter author have resulted in its discovery in San Francisco Bay, in Tomales Bay, and at Balboa Point; and both sexes yielding eggs and sperms have been maintained for months in the laboratory where also the larvae have been successfully reared through metamorphosis. This exceptional viability under laboratory conditions makes this marine animal especially suitable for many sorts of experimental studies.

During the course of the present investigation, covering a period of about 5 months, fresh supplies of the worms were provided every week or 10 days.[1] The sexes were kept separate in aquaria into which a forceful jet of water carrying air was constantly supplied. Eggs or sperms were removed, as needed, according to MacGinitie's method of carefully inserting a slender pipette into one of the gonopores, which usually afforded a copious supply. The eggs were placed in a finger bowl which contained fresh, filtered sea water. They were washed and their fertility was tested before being transferred to the centrifuge tubes. Only freshly obtained eggs or sperms were used throughout the experiments.

[1] I am indebted to Professor G. E. MacGinitie for supplying all of the animals for these experiments, and for other generous aid, and to Miss Vesta Holt, Mr. H. Hess, and Mr. R. Bolin for technical assistance.

Following their removal from the centrifuge tubes, the eggs were washed with fresh sea water and inseminated immediately or after a given period of time. With rare exceptions the centrifuged eggs took membranes and developed at a rate approximately equal to that of normal eggs. Their development was studied in Syracuse dishes or, with higher magnification, on hanging drop slides.

In order to ascertain accurately the position of polar bodies with respect to the axis of stratification for large numbers of eggs, it was found necessary to transfer them upon the appearance of the polar bodies to a cold chamber which maintained a temperature of about 6° C. on the stage of the microscope. The rectangular cold chamber, in size $11 \times 6 \times 1\frac{1}{2}$ cm., was made of copper sheeting so as to form a box with continuous hollow walls which were well insulated with asbestos and, over the asbestos, with several coatings of Duco. The Duco, when thoroughly dried, is apparently non-toxic, and renders the chamber useful for high, as well as for low, temperature experiments. A microscope slide, sealed in place with the Duco, served as the floor of the chamber proper, which was $7\frac{1}{2} \times 2\frac{1}{2} \times 1\frac{1}{4}$ cm., with a capacity of about 23 cc.

For locating the position of polar bodies, the matured eggs were evenly scattered over the floor of the cold chamber in 20 cc. of fresh sea water. Brine water from a storage tank having an initial temperature of $-18°$ C. was circulated throughout the hollow walls of the cold chamber by means of a centrifugal pump at a controlled rate such as to maintain a temperature in the cold chamber of about 6° C. during the period of observation.

This procedure proved to be very useful also for a prolonged, detailed study of the visible changes within the living egg during its maturation, fertilization, and early cleavages. The egg's rate of development at 6° C. was retarded about eight times that at room temperature (17° C.).[1]

Samples of eggs from each of the experimental lots were fixed and stained either entirely or after sectioning. Two killing reagents, of the fifteen that were tried, proved the most satisfactory. For the fertilization membrane and polar bodies, a chromic-formol-acetic reagent (Guyer, 1930) was best; while Zenker's, which was made up

[1] This determination was made by Mr. G. Torreblanca.

with sea water, appeared to be the best fixative for general use for both eggs and larvae. In either solution, the eggs were fixed for 1 hour and washed 48 hours in running sea water and distilled water, followed by very gradual dehydration.

The eggs were stained in iron-haematoxylin and, in some instances, counterstained with acid fuchsin or orange G or both.

The centrifuge employed in all of the experiments here reported was the Haematokrit model manufactured by Bausch and Lomb. Its revolutions per minute tested 8,700 during experimental operations, and its centrifugal force was computed to be approximately 4,800 times gravity. The centrifuge was operated on the city current, the supply of which varied to a slight but unknown extent.

The volume of eggs and of sea water in each of the two centrifuge tubes was practically the same for all experiments.

The sea water for each experiment was taken directly from the Bay and filtered through new and unused filter paper. Variations in its solutes, although probably significant, were not determined.

All glassware was kept scrupulously clean and free from fumes of reagents or other toxic substances.

The temperature of the centrifuge was fairly well controlled by running the sea water from the general supply tank through a $\frac{1}{2}$-inch spiral copper tube of ten turns which was fitted just inside of the bowl of the centrifuge. Temperature readings were recorded every half-hour during the experiment, at which time the centrifuge was oiled.

The maximum variation of temperature readings was 2.4° C. (15.8°–18.2°). The greatest variation in temperature during an experiment was 1.1° C. (17.0°–18.1°), and the least such variation was 0.2° C. (16.5°–16.7°). Under the caption, "The Centrifuged Egg," the amount of temperature variation is noted for each experiment.

THE NORMAL EGG

The eggs of *Urechis caupo* are shed in the germinal vesicle stage and normally remain in that stage until fertilized. These immature eggs are readily distinguishable also by a marked depression that occupies about 21 per cent of the total volume of the egg. More than one depression occurs in some eggs. It is found in eggs within the

gonoduct as well as in those that have been shed into the water. Normally it does not disappear until after fertilization. Eggs were kept in filtered sea water at room temperature (15°–17° C.) for 50 hours without the loss of the germinal vesicle or the disappearance of the depression, and upon insemination about 90 per cent took membranes and underwent fairly normal development.

The average diameter of 303 eggs which were selected at random from batches taken from 29 females was 128.32 μ. Their diameter varied from 123.55 μ to 144.73 μ.

Individual eggs are fairly transparent. The eggs *en masse*, however, are commonly pink, owing to the inclusion of numerous red granules which are dispersed throughout the cytoplasm. There occur also yellow, brown, and black inclusions. The red granules vary in number, and may be almost wanting, in eggs from some or all of the 6 gonoducts of some females. Such egg masses are then yellow or brown.

The conspicuous germinal vesicle is usually displaced to some extent by the depression. It measures 58 μ in diameter and contains one nucleolus which is 12 μ in diameter. The latter is supported by hyaline strands (Fig. 1) a few of which can be traced to the wall of the vesicle. When eggs are caused to burst by gradual pressure on the cover slip, the germinal vesicle may occasionally be seen to pass out into the surrounding water where it has remained intact for several minutes. When it swells and bursts, the nucleolus may in turn be observed to pass into the water. It remained there in one instance for about 10 minutes before its swelling brought about its complete disappearance.

The immature egg apparently has no mark of polarity. Neither the eccentricity of the germinal vesicle nor the position of the one or more depressions could be correlated with the subsequent place of extrusion of the polar bodies, as will be stated in some detail in later paragraphs (see p. 433).

Observations on fertilization were made chiefly on the living egg. These have had to do more especially with what, if any, relation the entrance point of the sperm might have with the place of origin of the polar bodies and with the direction of the first plane of cleavage.

In a dilute sperm suspension the eggs, when mounted in a hanging

drop, show clearly the entrance path of the sperm, which path remains conspicuous for several minutes in the swelling fertilization membrane. This observation was greatly enhanced by mounting a few eggs just after insemination in a hanging drop of 0.2 per cent agar–sea-water solution. By this means the entrance path through the membrane of as many as 10 eggs in the same field has been observed and mapped under a magnification of 120×, and the subsequent position of the polar bodies and the first plane of cleavage definitely ascertained.

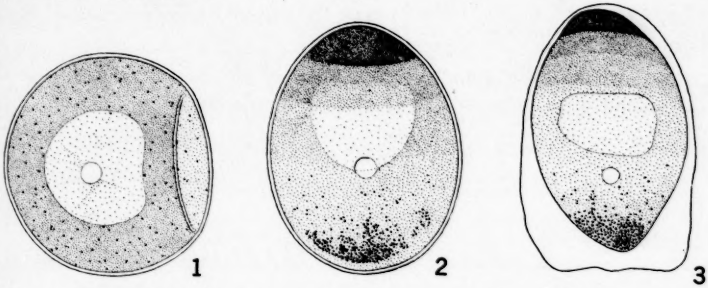


FIG. 1.—Normal immature egg showing the depression and the general distribution of red granules and other inclusions.

FIG. 2.—Immature egg centrifuged 8 hours. At the upper, centripetal pole appear the "black cap," and the brown and the yellow strata. The nucleolus which has been displaced toward the centrifugal pole now lies in a pocket depression of the membrane of the germinal vesicle. Most of the red granules are clustered toward the outer (centrifugal) pole, yet do not form a definite stratum.

FIG. 3.—A fertilized egg which had been centrifuged 10 hours. The three layers of black, brown, and yellow inclusions are clearly defined. The nucleolus lies completely outside of the vesicle to which the former was not visibly attached. The red granules are not all confined near the centrifugal pole and again are not stratified. The much greater thickness of the fertilization membrane on the centrifugal side of the egg is characteristic.

The data indicate that the sperm may enter at any point on the egg's surface irrespective of the place of origin of the polar bodies. There is a marked tendency, however, for the first cleavage plane to pass through or near-by the entrance point of the sperm. In 26 eggs, the cleavage plane of 18 passed directly through the sperm's entrance point; in 4 this plane passed very near that point; while in in the remaining 4 the entrance point was as much as 90° from this

plane of cleavage. Although dispermy occurs in some eggs, it could not be seen in any of the 26 eggs noted above, including the last 4, in which a discrepancy between entrance point and the first cleavage plane was most marked.

In a later paragraph it will be seen that these relations of the entrance point of the sperm and the position of the first plane of cleavage hold for centrifuged eggs about the same as for normal ones. In this connection, however, one fact is worthy of special emphasis, viz., that the sperm rarely, if ever, enters the region of the "black cap" which appears so distinctly in eggs that have been centrifuged for 5 hours or longer.

Table I[1] lists the general distribution of the points of entrance of the sperm with reference to the poles, hemispheres, and equator, in

TABLE I

DISTRIBUTION OF POINTS OF ENTRANCE OF SPERM

	I	II	III	Total
Animal pole	1	1	0	2
Animal hemisphere	5	5	7	17
Equator	6	5	3	14
Vegetal hemisphere	3	4	5	12
Vegetal pole	4	4	4	12
Total	19	19	19	57

a total of 57 eggs which comprised 19 eggs from each of 3 females. It is clear from this table that the sperm may enter the egg of *Urechis caupo* at any point on its surface. The number of entrance points at the vegetal pole was six times the number at the animal pole; but since the number of entrances in the animal hemisphere was somewhat greater than those in the vegetal hemisphere, the larger percentage recorded for the vegetal pole cannot have much, if any, significance, especially when the total number of eggs observed was relatively meager.

As stated in a foregoing paragraph, the newly shed eggs of *Urechis caupo* are immature and their maturation normally does not ensue until after the entrance of the sperm. From 32 to 40 minutes thereafter, at about 17° C., the first polar body appears. At that tempera-

[1] From studies by Mr. H. B. Hess.

ture, the second polar cell is given off approximately 17 minutes later beside the first.

The extrusion of polar bodies in this egg is a conspicuous and impressive phenomenon. With the eggs mounted in a shallow hanging drop of the agar–sea-water solution noted above, the entire process of extrusion of the polar cells may be observed under the oil immersion lens. This affords a view of the Brownian movement of particles and the surges of larger inclusions such as may hardly be equaled in any other living cell.

After extrusion, each polar body tends to flatten against the periphery of the egg. Their major and minor axes then measure about 9 μ and 6 μ, respectively. The first polar cell occasionally undergoes division. Save for the nucleus, each of the polar bodies is optically empty. Their non-granular cytoplasm distinguishes them from other extrusions which may sometimes appear on both normal and centrifuged eggs.

The position of formation of polar bodies is, as formerly noted, apparently unpredictable in the immature egg. Their place of origin bears no known relation either to the point of entrance of the sperm or to the eccentric position of the germinal vesicle or to the depression.[1]

Some conspicuous mark of polarity, such as the depression or the eccentricity of the germinal vesicle, would have been exceedingly useful in the centrifuging experiments on immature eggs; and, accordingly, especial search was made to that end.

The agar hanging-drop method described above was used in comparing the position of the depression in the immature egg with that of the later origin of polar bodies in the fertilized egg. The results given in the following table show that in the 43 eggs thus examined no correlation existed between the relative positions of polar bodies and the depression:

	Eggs
Polar bodies at center of depression	3
Polar bodies within depression but not central	13
Polar bodies just outside of depression	8
Polar bodies in hemisphere opposite depression	19
Total	43

[1] Determinations made by Miss Vesta Holt.

Also, a comparison was made between the eccentric location of the germinal vesicle and the place of extrusion of the polar bodies. While the eccentricity of the germinal vesicle appeared to be due to its displacement by the depression, it was assumed, nevertheless, that its actual position might bear some relation to the point of origin of the polar cells and so indicate the direction of the polar axis in this egg, as is the case in many other immature ova. Moreover, the contents of the germinal vesicle flow somewhat amoeba-like during maturation, toward the place of extrusion of the polar bodies; and the direction of such flow might very well be toward that point on the periphery which is nearest the germinal vesicle after the depression has filled out.

It was found possible to induce artificially the disappearance of the depression by subjecting the immature eggs to hypotonic sea water. Thereupon they were imbedded in agar, the eccentric positions of the germinal vesicle were mapped, and the spermatozoa were added. The relative locations of the polar bodies were then recorded. For four such experiments, the results were as follows:

	Eggs
Polar bodies at point nearest germinal vesicle	1
Polar bodies in hemisphere containing germinal vesicle	9
Polar bodies located "equatorially"	8
Polar bodies in hemisphere opposite germinal vesicle	9
Polar bodies at point farthest from germinal vesicle	2
Total	29

This experiment was difficult and the results need further checking. It appears fairly certain, however, that the germinal vesicle in the eggs of *Urechis caupo* lies indifferently with respect to the direction of the polar axis as indicated by the point of extrusion of the polar cells.

It was further supposed that the immature egg might have a heavier side due to an unequal distribution of cytoplasmic inclusions. This possibility had special significance in the results of centrifuging, and so it was carefully checked by three simple experiments:

1. A rectangular glass chamber, $5 \times 5 \times \frac{1}{2}$ inches, filled with sea water, was supported vertically in the field of a binocular. Unferti-

lized eggs were dropped with care into the chamber, and their descent through the water was observed by means of the binocular. It would be expected that immature eggs would descend with the depression uppermost, and such is the case with rare exceptions. Fertilized eggs in which the depression had just disappeared were, therefore, substituted. They were observed to give off polar bodies some minutes after they had reached the bottom of the chamber, whereupon the positions of the polar cells with reference to the upper and lower halves for 102 eggs, in a total of four counts, were recorded as follows:

	Eggs
Polar bodies in the upper half	48
Polar bodies in the lower half	54
Total	102

Similarly, eggs in the 2-cell stage were allowed to fall through the water. During their descent, as well as after the eggs had reached the bottom of the chamber, their plane of cleavage was observed to maintain any and all possible positions with respect to the vertical.

2. Immature eggs were allowed to settle to the bottom of a test tube filled with sea water. Sperms were then added, and at the proper time a vaselined cover slip was held tightly over the mouth of the tube while the latter was inverted over a 1-inch Syracuse dish completely filled with ungelled agar. The cover glass was in a few cases successfully removed, so that the fertilized, fully rounded-out eggs settled quietly upon the agar and became imbedded in it.

The positions of the polar bodies of 237 of the imbedded eggs were observed as follows:

	Eggs
Polar bodies on the upper half	90
Polar bodies near the "equator"	51
Polar bodies on the lower half	96
Total	237

3. Fertilized eggs were dropped from a pipette into a Syracuse dish containing sea water. The dish was left undisturbed, and the positions of the polar bodies noted. Following are the results of four counts:

	Eggs
Polar bodies on upper half	100
Polar bodies near "equator"	58
Polar bodies on lower half	81
Total	239

From these consistent results, it appears to be conclusive that in the eggs of *Urechis caupo* the materials are distributed evenly throughout the cytoplasm, and so there is no heavier side.

Early cleavage in *Urechis caupo* appears to conform well with that of most yolk-poor eggs. The first cleavage plane, in all eggs carefully studied, passed quite or almost exactly through the polar axis as defined by the position of the polar bodies. In several instances the two polar cells were separated by this plane of cleavage, leaving a polar body attached to each blastomere. The second cleavage plane also passes through the axis at right angles to the first, and the third plane of cleavage is equatorial. The blastomeres are apparently adequal up to the 16-cell stage. The archenteron is formed by invagination, and the blastopore soon becomes relatively quite narrow.

The trochophore larva bears an apical tuft of a few very long cilia and a band of preoral cilia. At room temperature (17° C.) the early trochophore stage may be reached in about 16 hours after the first cleavage.

A striking behavior of the red granules during early cleavage was first observed in eggs that had been centrifuged 3 hours. This behavior was then seen also in the uncentrifuged egg, and apparently is constant for this species. During the formation of the amphiaster, the red granules, which in immature eggs are dispersed throughout the cytoplasm, come to be aligned irregularly along the astral rays. They then migrate down the rays to become concentrated about the daughter-nucleus in the form of a dense ring. This same behavior was observed in a number of dividing cells of the 16-cell stage, so that it seems probable that these granules are fairly equally distributed to each of the cells at least during early development. Their behavior is very evident in dividing centrifuged eggs where other cytoplasmic inclusions do not obstruct the view. There the direction of the first plane of cleavage may be such that all of the red granules pass to one blastomere. This, however, apparently does not influence

the normal course of development. Under oil immersion, these red granules exhibit Brownian movement before and during their migration.

THE CENTRIFUGED EGG

When a centrifugal force of 4,800 times gravity is applied to the immature egg of *Urechis caupo* for 3 hours or longer, the cell contents become stratified into five fairly well-defined zones. It will be convenient, however, to regard these zones as demarcating the centrifuged egg essentially into hemispheres. One of the hemispheres, the centrifugal, contains mostly hyaline protoplasm in which are embedded many red granules that tend to cluster toward the centrifugal pole. There may appear also near this pole, especially in eggs centrifuged from 7 to 10 hours, an indefinite grayish mass, which, however, is not evident in some eggs. The other, centripetal hemisphere is more or less opaque owing to the stratification there of yellow, brown, and black inclusions. The latter become condensed at the centripetal pole into a crescentic black cap (Fig. 2).

Accordingly, the centrifugal hemisphere will be referred to as the "hyaline" hemisphere, the centripetal as the "opaque" hemisphere, and their respective poles as the "red" and the "black" pole. The composition of these several stratified inclusions in this egg is not known. The black stratum, being the lightest, may very likely be oil; but all of the strata of this opaque hemisphere are readily stainable with Sudan III, and they are densely blackened by osmic acid. If any of these three layers is yolk then in this egg the yolk is lighter than the hyaline protoplasm, which is unusual.

The germinal vesicle migrates centripetally, and in eggs of longer periods (6–10 hours) of centrifuging this vesicle becomes markedly flattened against the strata of the centripetal pole. The single nucleolus, on the contrary, moves toward the centrifugal pole; and during 8 or 10 hours of centrifuging it may be thrown completely outside of the germinal vesicle, to which it may or may not remain attached by a hyaline strand. The extraordinary behavior of some of these expelled nucleoli during fertilization will be described later on.

It should be emphasized that the red and the black inclusions behave very differently under centrifugal force. The latter tend always to concentrate into a clearly defined stratum—the crescentic

black cap which may become completely pinched off during the longer runs. The red granules, on the other hand, never form a true stratum but, instead, cluster into one, two, or several irregular masses; or, in many centrifuged eggs of a given lot, they may be found very unevenly dispersed throughout the greater part of the hyaline hemisphere. It is possible that this chaotic variation in their distribution depends upon distortion of the egg from pressure of surrounding eggs. But this distortion might be expected to affect also the black inclusions, which apparently is not the case. At any rate, it is evident that these two sorts of inclusions exhibit strikingly different physical properties, a fact which, as we shall see later on, has a direct bearing upon the interpretation of the general results of these centrifuging experiments.

It will again be recalled that freshly shed eggs of *Urechis caupo* are immature and that the polar cells are normally not given off until after the sperm has entered the egg. As in the normal egg, it appears that the sperm may enter at any point on the centrifuged egg's surface, with a noteworthy exception, viz., that the sperm rarely, if ever, enters the region of the black cap at or near the centripetal pole. Of 15 centrifuged eggs, 2 showed the sperm entering at the red pole, 8 showed its entrance into the hyaline hemisphere, 4 into the opaque hemisphere, and 1 egg had the entrance point at the black pole, but this egg did not develop.

Membrane elevation on centrifuged eggs exhibits one striking contrast to that on the normal egg: apparently irrespective of the point of entrance of the sperm, the fertilization membrane of the centrifuged egg tends to appear first in the region of the red (centrifugal) pole. Moreover, the fully formed membrane is usually several times wider on that side of the egg (Fig. 3).

The disappearance of the germinal vesicle in the fertilized centrifuged egg occurs at about the same time as was noted for the normal egg. The vesicle which is always displaced toward the centripetal pole, during the maturation process moves to the center of the more or less rounded-up egg shortly before the vesicle disappears. It was previously mentioned that during long periods of centrifuging the nucleolus which migrates toward the centrifugal pole may be expelled completely outside of the germinal vesicle to which

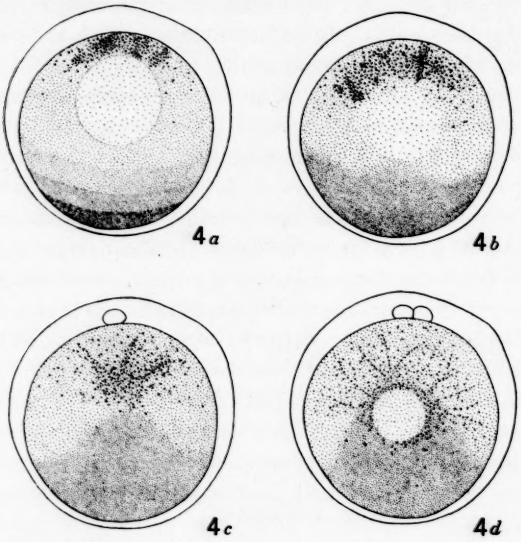
it may or may not remain attached (Fig. 3). Its behavior in a few such cases was studied during maturation when, in each of four recorded cases, the nucleolus gradually returned to the interior of the vesicle. In one instance, this occurred and yet no strand connecting the nucleolus and vesicle could be detected.

The extrusion of polar bodies will be given special consideration in later paragraphs, since their place of origin with respect to the axis of stratification is of major interest in these centrifuging experiments. Mention may be made here, however, of the fact that their manner and time of extrusion obtains essentially the same for centrifuged as for normal *Urechis* eggs. It was noted in describing the cleavage of the normal egg that the red granules tend to migrate down the astral rays of the cleavage spindle. This phenomenon of migration of the red granules may be observed under especially suitable conditions to occur to some extent also during maturation in the centrifuged egg. It apparently may happen, therefore, whenever the granules are disposed near the astral rays. The size of polar bodies in the centrifuged egg, with one notable exception, is approximately normal. This exception, to which further attention will be given, concerns polar bodies that may infrequently be given off in the region of the dense black cap. Here they are much smaller, since their diameter may be not more than one-eighth that of polar bodies (of normal size) extruded elsewhere on the centrifuged egg.

The cleavage process in centrifuged eggs is also comparable with that in the normal egg. The first and second cleavage planes, with no observed exceptions, pass through or very near the place of origin of the polar bodies. The third cleavage plane is equatorial. Some attention was given to the relation of the plane of first cleavage to the entrance point of the sperm in the centrifuged egg. The data, as far as it goes, indicate that this plane of cleavage here, as in the normal egg, tends to pass through or near-by the entrance point.

It was observed that in the dividing centrifuged egg one end of the amphiaster becomes deformed or, at times, apparently unformed, when that end approaches near or abuts against the stratified layers of the opaque hemisphere. This fact appears to have a distinct significance which will be discussed farther on.

The remarkable migration of the red granules during maturation and cleavage has already been described; but it may here be further stated that in the centrifuged egg (Figs. 4*a–d*), where this phenomenon was first observed, this interesting behavior is particularly visible in the region of the hyaline hemisphere. If, as sometimes happens, the first cleavage plane passes at right angles to the axis of



FIGS. 4*a–d*.[1]—Stages during maturation which show especially the behavior of the red granules in their migration down the astral rays of the developing first-cleavage spindle.

stratification, then obviously all of the red granules come to be included in one blastomere. In a few (6) recorded instances this blastomere divided several minutes before the one which contained the dark inclusions. In as many or more eggs this did not obtain, and so the question remains as to whether or not these granules affect in any definite manner the rate of cleavage or the normality of development (but cf. Whitaker, 1929).

Record should be made of one striking tendency in the first and second cleavages of the centrifuged eggs which was especially

[1] Drawn by Mr. R. Bolin.

marked when these cleavage planes passed approximately through the axis of stratification. It could then be clearly seen that the constriction progressed far into the hyaline hemisphere (Fig. 5) before it made its appearance in the black cap of the opaque hemisphere (cf. Whitaker, 1929). This tendency will be later referred to in the interpretation of the general results.

Gastrulation in the centrifuged eggs of *Urechis* appeared to differ in no essential from that in the uncentrifuged egg. The observations thus far indicate that, as in the normal larvae, invagination here takes place diametrically opposite to the position of the polar bodies irrespective of the disposition of the stratified materials. This would mean, then, that the place of origin of the polar bodies also in the centrifuged egg defines the direction of the polar axis.

These relationships appear to hold without exception for the trochophore larvae arising from the centrifuged eggs (Figs. 6*a*, *a'*–*d*, *d'*). Further attention to this point will be given in later paragraphs.

We may now return to a consideration of the origin of polar bodies in these centrifuged eggs with special reference to any relation their place of origin may have to the axis of stratification. In eggs which had been centrifuged 5 hours, it was first observed that apparently a large majority of polar cells had been extruded from the hyaline hemisphere and many of these appeared at or near the centrifugal (red) pole. A count revealed that the relative number of polar bodies occurring on this hemisphere and pole amounted to about 68 per cent. An extensive investigation was then made of this phenomenon, and a major part of this report is devoted to an account of the results obtained.

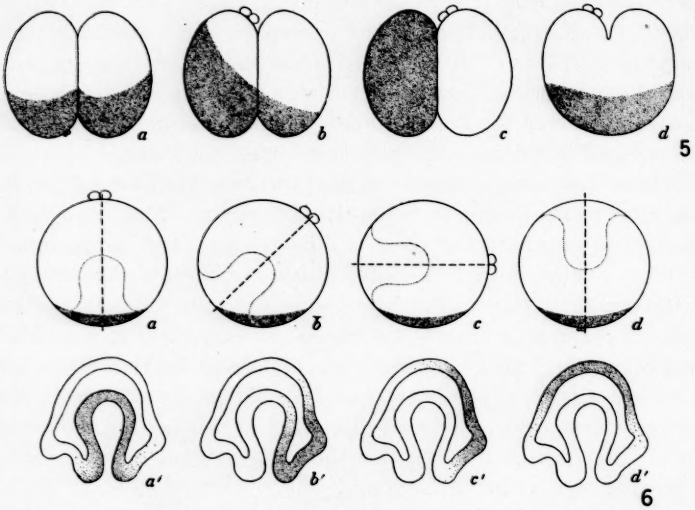
Throughout the centrifuging experiments that followed, the strength of force applied was approximately 4,800 times gravity. The periods of duration of this applied force covered a range for each hour from 1 to 12, and one lot of eggs was centrifuged for 18 hours.

The number of lots of eggs centrifuged for each of the hour-periods varied from two for the 4-, 5-, 9-, 10-, and 11-hour periods to six lots for the 7-hour period. Three lots of eggs were centrifuged for each of 2, 3, and 12 hours, and four lots for 1, 6, and 8 hours, respectively.

For these twelve periods of duration, the group total of eggs of which the relative positions of polar bodies were carefully recorded

varied from 434 eggs of the 11-hour period to 2,092 of the 7-hour period. The sum total of all centrifuged eggs thus recorded was 14,045.

For recording the relative positions of polar cells on these centrifuged eggs, five regions were arbitrarily assigned, as follows: (1) the red pole; (2) the hyaline hemisphere; (3) the equator, in width about the diameter of a polar body; (4) the opaque hemisphere; and (5)



FIGS. 5*a–d*.—Diagrams representing four possible dispositions of the black, brown, and yellow inclusions of the "opaque hemisphere" with reference to the polar axis. Figure *d* illustrates the beginning of the constriction of the hyaline cytoplasm which regularly occurs whenever the centripetal and vegetal poles coincide.

FIGS. 6*a–d* AND *a'–d'*.—Diagrams of centrifuged eggs and their subsequent gastral and larval stages. With respect to the polar axis, the same relative disposition of the material of the black cap tends to persist throughout at least early larval development.

the black pole. At first an ocular micrometer was used in delimiting these several regions, but after considerable practice the micrometer was considered unnecessary and so was dispensed with. It may be noted that as a test of accuracy in these observations the record of a second sample of a given lot of eggs not infrequently checked to within less than 1 per cent of the record of the first sample.

A complete summary of the records made of the regional positions

of polar cells, as indicated above, is given in the general table (Table II). It will be observed that this table lists in serial order each lot of eggs centrifuged for periods successively from 1 to 12 hours, and the single lot which was centrifuged for 18 hours. In the ten columns there appear for each lot the number and percentage of eggs having polar bodies (1) at the red pole, "R.P."; (2) on the hyaline hemisphere "H.H."; (3) the sums of (1) and (2); (4) at the equator; (5) the sums of (6) and (7); (6) on the opaque hemisphere "O.H."; (7) at the black pole "B.P."; (8) the total eggs recorded for each lot; (9) total eggs recorded for each period; and (10) temperature variations during each run of the centrifuge.

For general comparisons of the results, columns (3), (4), and (5) may be conveniently referred to. For the four lots of eggs of the 1-hour series, column (3) shows that the percentage of eggs having polar bodies on the hyaline hemisphere and red pole ranged from 45.7 to 50.8 per cent, while the percentage of those on the opaque hemisphere and black pole (col. 5) varied between 38.6 and 40.7 per cent.

Passing down column (3), a comparison of percentages throughout the list indicates a general trend that seems unmistakable, viz., that there is a progressive increase in the relative percentage of polar bodies occurring on the hyaline hemisphere and red pole which tends to vary directly with the increase of duration of the applied centrifugal force. Also, a corresponding decrease in the percentage of polar bodies in the opaque hemisphere and black pole is evident upon comparing the successive percentages down column (5).

Upon closer examination, however, it will be seen that, especially in adjacent periods, there is a considerable overlapping of percentages. One 3-hour lot (No. 10), e.g., shows only 46 per cent of polar cells on the hyaline hemisphere and red pole, which is a lower percentage than that (52.9 per cent) of lot No. 5 of the 2-hour series and even lower than the percentage (50.8) of lot No. 4 of the 1-hour runs. Similar discrepancies appear throughout the list. Of the 6-hour series, e.g., lot No. 15 shows only 62.5 per cent of polar bodies on the hyaline hemisphere (and pole), which approximates the percentage (66.1) for lot No. 30 of the 9-hour runs and that (67.3 per cent) of the 11-hour lot No. 33.

TABLE II*

Summary of Regional Positions of Polar Bodies

Lot No.	Length of Period (Hours)	(1) R.P.		(2) H.H.		(3) R.P.+H.H.		(4) Equator		(5) O.H.+B.P.		(6) O.H.		(7) B.P.		(8) Total Eggs for Each Lot	(9) Total Eggs for Each Period	(10) Temperature Variations during Each Run of Centrifuge (Centigrade)
		No.	Per-centage	No.	Per-centage	No.	Per-centage	No.	Per-centage	No.	Per-centage	No.	Per-centage	No.	Per-centage			
1	1	13	4.9	120	45.6	133	50.5	24	9.1	106	40.4	96	36.5	10	3.9	263		17.0–17.6
2	1	5	4.4	47	41.2	52	45.7	20	17.6	42	36.8	35	30.7	7	6.1	114	1,013	17.0–17.2
3	1	7	2.5	128	46.0	135	48.5	33	11.9	110	39.6	102	36.7	8	2.9	278		17.8–18.0
4	1	15	4.2	167	46.6	182	50.8	41	11.4	135	37.8	129	36.1	6	1.7	358		17.2–18.0
5	2	11	2.4	231	50.5	242	52.9	48	10.4	168	36.7	162	35.4	6	1.3	458		17.4–18.1
6	2	9	2.9	147	48.0	156	50.9	36	11.7	115	37.4	111	36.1	4	1.3	307	1,194	17.6–17.8
7	2	18	4.2	211	49.2	229	53.4	46	10.7	154	35.9	144	33.6	10	2.3	429		17.2–17.8
8	3	10	3.0	169	51.4	179	54.4	41	12.5	109	33.1	103	31.3	6	1.8	329		17.8–18.1
9	3	23	7.6	133	43.8	156	51.4	45	14.8	103	33.8	91	29.9	12	3.9	304	855	17.6–18.0
10	3	17	7.7	85	38.3	102	46.0	42	18.9	78	35.1	64	28.8	14	6.3	222		17.0–17.8
11	4	21	2.9	386	54.3	407	57.2	57	8.0	247	34.8	243	34.2	4	0.6	711	1,378	17.0–18.1
12	4	27	4.1	330	49.5	357	53.6	75	11.2	235	35.2	233	34.9	2	0.3	667		17.2–18.0
13	5	14	2.6	291	53.3	305	55.9	61	11.2	180	32.9	178	32.6	2	0.3	546	1,116	17.0–17.5
14	5	11	2.0	310	54.4	321	56.4	57	11.0	192	33.6	188	32.9	4	0.7	570		17.1–17.5
15	6	11	6.3	103	58.9	114	65.2	16	9.1	45	25.7	43	24.6	2	1.1	175		17.2–18.0
16	6	15	11.0	65	50.8	80	61.8	18	14.0	30	23.5	29	22.7	1	0.8	128	1,392	17.0–17.8
17	6	33	5.8	287	50.8	320	56.6	91	16.0	156	27.4	150	26.4	6	1.0	567		17.1–17.6
18	6	34	6.5	257	49.2	291	55.7	85	16.3	146	28.0	143	27.4	3	0.6	522		17.2–17.6
19	7	33	11.0	136	45.3	149	56.3	45	15.0	93	28.7	82	27.4	4	1.3	300		17.0–17.6
20	7	13	10.7	62	50.8	75	61.5	19	15.6	28	22.9	27	22.1	1	0.8	122		17.2–18.0
21	7	12	3.6	182	54.5	194	58.1	51	15.2	89	26.7	84	25.2	5	1.5	334	2,092	17.0–17.8
22	7	27	5.2	277	52.7	304	57.9	93	17.7	128	24.4	122	23.3	6	1.1	525		17.2–17.6
23	7	27	7.4	190	52.1	217	59.5	57	15.6	91	24.9	88	24.1	3	0.8	365		17.0–17.8
24	7	19	4.3	246	55.2	265	59.5	80	17.9	101	22.6	99	22.2	2	0.4	446		17.4–18.0

* This table lists in serial order each lot of eggs centrifuged for each hourly period of from 1 to 12 hours and one lot (2 counts) of an 18-hour period.

TABLE II—*Continued*

25	8	38	9.2	252	61.0	290	70.2	49	11.9	74	17.9	72	17.4	2	0.5	413	1,214	17.6–18.1
26	8	17	5.6	194	63.2	211	68.8	43	14.0	53	17.2	50	16.2	3	1.0	307		17.6–18.0
27	8	28	9.3	185	61.4	213	70.7	36	12.0	52	17.3	51	16.9	1	0.4	301		17.8–18.2
28	8	18	9.3	118	61.1	136	70.4	20	10.4	37	19.2	36	18.7	1	0.5	193		17.0–17.7
29	9	36	4.9	485	67.2	521	72.1	77	10.7	124	17.2	123	17.0	1	0.2	722	1,528	17.2–18.0
30	9	46	5.7	487	60.4	533	66.1	99	12.3	174	21.6	172	21.4	2	0.2	806		16.5–16.7
31	10	21	7.9	167	62.5	188	70.4	28	14.9	51	19.2	49	18.4	2	0.8	267	520	17.4–18.0
32	10	28	11.1	148	58.5	176	69.6	30	11.9	47	18.5	44	17.4	3	1.1	253		17.1–17.8
33	11	19	7.4	153	59.9	172	67.3	39	15.2	45	17.5	42	16.4	3	1.1	256	434	15.8–16.8
34	11	18	10.1	102	57.3	120	67.4	25	14.0	33	18.6	30	16.9	3	1.7	178		16.8–17.6
35	12	28	9.3	195	64.8	223	74.1	20	6.7	58	19.2	57	18.9	1	0.3	301	744	16.4–16.8
36	12	26	10.3	157	62.3	183	72.6	21	8.3	48	19.1	46	18.3	2	0.8	252		17.2–17.8
37	12	22	11.5	118	61.7	140	73.3	14	7.3	37	19.5	36	18.9	1	0.8	191		16.8–17.4
38	18	28	10.5	152	57.1	180	67.6	31	11.6	55	20.8	53	20.0	2	0.8	266	565	16.8–17.4
39	18	26	11.3	135	58.9	161	70.4	19	8.3	49	21.5	46	20.2	3	1.3	299		16.8–17.4

No explanation can be offered for these and other discrepancies that appear here and there throughout the general table. The range and degree of temperature differences during centrifuging may have been effective in the 9-hour lot (16.5°–16.7° C.) and the 11-hour lot (15.8°–16.8°), as against (17.1°–18.0°) for the 6-hour lot referred to in the preceding paragraph. But a comparison of temperature differences and the percentage of polar bodies for lot No. 35 (16.4°–16.8° and 74.1 per cent) and lot No. 37 (16.8°–17.4° and 73.3 per cent), both of which are of the 12-hour series, would indicate, if anything, that the higher temperature gave a lower percentage of polar bodies on the hyaline hemisphere and red pole. Further reference to these discrepancies appears under the subsequent caption "Discussion."

It should be noted that record was made of all eggs observed in each lot listed in the general table. This included not only (1) all eggs having clearly defined polar bodies but also (2) eggs on which polar bodies could not be found, (3) eggs without membranes, (4) cytolyzed eggs, and (5) those with polar cells the position of which could not be accurately determined. This last group (5) comprised eggs which were not clearly stratified or whose orientation in the microscopic field was sometimes such as to obscure a clear view of the polar bodies without jarring the chamber, which might in turn disarrange the eggs under observation.

It can be said further, however, that while it seemed unnecessary to include here the percentages of these several kinds of bad eggs, special precautions were taken so that in all of the lots here reported these percentages were considered to be negligible, with one exception, viz., the lot (two counts) of the 18-hour series. This contained a very considerable number of cytolyzed eggs, estimated to be as much as 10 or 15 per cent. But since only one lot of eggs was centrifuged for this long period, the results were included, as shown in the general table. Record was made of three lots of centrifuged eggs, of a 3-hour, a 9-hour, and a 12-hour run, which were discarded because too many were cytolyzed.

Especial mention should be made of the marked tendency of the stratified inclusions gradually to redisperse in these centrifuged eggs when they were allowed to remain uninseminated for any considerable time. This tendency, which has been recorded for other cen-

trifuged eggs (cf. Conklin, 1917), proved here to be advantageous as a means of testing the validity of the interpretation of the general results which are to be discussed in later paragraphs. It was previously noted that the lots of centrifuged eggs, unless otherwise specified, were all inseminated immediately upon their removal from the centrifuge in order to insure results as uniform as possible. The 39 lots, as summarized in Table II were all thus promptly inseminated.

There were three other lots of similarly centrifuged eggs, however, in which insemination was purposely delayed for several hours. The results which were recorded on the regional location of the polar cells will now be briefly given.

Lot A.—These immature eggs were centrifuged 5 hours and kept at room temperature (15°–17° C.) for 14 hours, whereupon they were inseminated with fresh sperms. The positions of polar bodies on 271 eggs of this lot were recorded as follows:

	Eggs
1. At red pole	6 (2.2%)
2. On hyaline hemisphere	126 (46.5%)
3. On equator	28 (10.3%)
4. On opaque hemisphere	109 (40.2%)
5. At black pole	2 (00.8%)
Total	271 (100.0%)

Lot B.—Eggs were centrifuged 7 hours and inseminated 14 hours later, under conditions noted for Lot A, above. On 247 eggs, the relative positions of polar bodies were:

	Eggs
1. At red pole	9 (3.6%)
2. On hyaline hemisphere	118 (47.8%)
3. On equator	21 (8.5%)
4. On opaque hemisphere	96 (38.9%)
5. At black pole	3 (1.2%)
Total	247 (100.0%)

Lot C.—Eggs were centrifuged 7 hours and kept for 22 hours at 11.0° C. They were then removed to room temperature for about 1 hour and inseminated thereafter. Many of the eggs failed to take membranes or cytolyzed (estimated to be about 10 per cent), but

the remainder extruded polar bodies normally, the positions of which were recorded as follows for 186 eggs:

	Eggs
1. At red pole	7 (3.7%)
2. On hyaline hemisphere	89 (47.9%)
3. On equator	11 (5.9%)
4. On opaque hemisphere	77 (41.4%)
5. At black pole	2 (1.1%)
Total	186 (100.0%)

The results of the foregoing three experiments are fairly consistent; and, as far as they go, they appear to indicate that along with a redispersion of the stratified inclusions of the egg an increasing number of polar bodies were extruded from the opaque hemisphere in which the strata had been confined. It is evident that along with this redispersion of inclusions there is correspondingly a return of the hyaloplasm more and more toward the centripetal pole. The significance of this will be discussed under the caption which now follows.

DISCUSSION

The experimental results of centrifuging the eggs of *Urechis caupo* as presented in foregoing paragraphs lack completeness because they are largely statistical and are confined exclusively to the immature ovum. Moreover, their value, so far as it concerns polarity, rests upon the assumption that the polar axis is defined in the centrifuged egg as well as in the normal egg by the place of origin of the polar bodies. Evidence supporting that assumption has also been presented.

It should be noted at the outset, however, that since no mark of polarity could be found in the immature ova of this species, it is not known that at this stage in their development a polar axis as yet exists. The applied centrifugal force could at most, therefore, be expected to influence the orientation of polarity as first visibly established in the fertilized egg. To what extent the data at hand indicate this effect in these experiments, we may presently undertake to examine.

It is obviously necessary to discuss these data in the light of the results of other similar studies and of some of the prevailing opinions

that have been maintained on the essential nature of polarity in the living cell. The phenomenon of polarity remains one of profound interest and importance since it appears to be a common property of all living cells and since in the living egg it becomes the primary mark of differentiation to which all further development tends to conform. Any facts or theories, therefore, which afford clearer insight into the nature of polarity are of fundamental value.

An essential problem of polarity was fully realized in the early work on centrifuging eggs. That problem had to do with the identification of the physical basis of polarity. Did polarity inhere in the cell as a whole? If so, was it a property of any and all visible parts and materials and structures of the cell? Or might there be some substances in the living cell which had little or nothing whatever to do with this property of polarity? The answer to these questions would do more than answer them, for it would contribute to the solution of some of the vexing problems on germinal localization, mosaic development, and other theories intimately akin to the parent theory of preformation. The behavior of certain inclusions in various eggs, such as the gray crescent of the frog's egg, the pink girdle around matured eggs of *Paracentrotus lividus* (Boveri, 1901), the yellow crescent in eggs of *Styela partita* (Conklin, 1905), in their behavior and fate might very plausibly represent formative or organ-forming substances as well as share an essential part in the polarity of the cell.

The experimental means of identifying the physical basis of polarity was provided by the centrifuge. When it was discovered that these visible inclusions in the egg could be shifted and stratified without impairing the normal development or modifying the original polarity of the cell, it became definitely established that the visible inclusions were not the material basis of polarity; neither were they organ-forming substances. It was obvious, then, that polarity and all formative processes throughout development were the common property of the hyaline, subsisting protoplasm—the "ground substance" (Lillie) or "spongioplasm" (Conklin).

If we inquire further into the nature of this ground substance or spongioplasm, the results of Conklin's studies (1917) identify it with the cortical layer of ectoplasm which also extends throughout the

cell as the internal framework that holds and connects the nucleus and the mitotic spindle and impales all inclusions of the cell. It is, according to Conklin, remarkably extensile and elastic, for in some of the centrifuged eggs the maturation spindle, which the spongioplasm incorporates, might be stretched enormously or thrown by the force applied the full diameter of the egg (to the vegetal pole), and yet by visible strands with which the spindle might remain attached to its original anchorage (the cortical layer at the animal pole), during subsequent development of the egg, this spindle would nearly or completely return to its normal location.

These properties of spongioplasm in the gasteropod egg have their counterpart in Lillie's (1906) "ground substance" in *Chaetopterus* eggs which was recognized as the seat of vital organization. The sensible fluidity of this basic substance must, according to Lillie, be compatible with a persistent, insensible structure, for, while this substance under the applied force readily yielded to the displacements of its various inclusions, the egg's subsequent development was normal and its original polarity and bilaterality remained unchanged. In view of this striking property of the ground substance, Lillie assigned to it a molecular basis of organization. The remarkable property of a fluid that may yet retain its essential structure concerns a physical state of matter generally which is now well recognized.

It is commonly conceded, then, that the visible inclusions of the cell which are subject to stratification under strong centrifugal force are not a necessary part of protoplasmic organization. Polarity, bilaterality, and the cyclic and formative processes of differentiation during development are independent of these grosser inclusions. It should be recalled, however, that the effectiveness of centrifugal force is a function of several limiting factors. As ordinarily defined, the resultant of that force acting in a given radius is equivalent to the product of the mass affected, the square of the angular velocity, and the period of total duration. For a given force applied to a heterogeneous system such as the cell, therefore, in unit time the degree of displacement of the component parts varies directly as their relative densities.

Then let us suppose that the ground substance is itself a hetero-

geneous system having components of quite dissimilar densities (cf. Conklin, 1929). In the terminology of the colloids this is an altogether plausible assumption. If, therefore, a centrifugal force applied to the ground substance were sufficiently great, it, too, would suffer a stratification of its colloidal components no less definite than that of its grosser, visible inclusions as effected by ordinary centrifuging. Indeed, in recent times many non-living colloidal systems have been successfully stratified by means of the ultra-centrifuge as perfected and employed by Svedberg (1928) and others. But to what extent the living ground substance would endure the rigors of such enormous forces (10,000–100,000 times gravity) and remain living, is, of course, exceedingly problematical. If the basis of protoplasmic organization is molecular—a postulate which now applies to colloidal systems generally—we may reasonably suppose that the living substance physically owes its being to the condition and maintenance of its unique structure. This qualification of the spatial relation of its component parts, once violated by mechanical or other forces sufficient to disrupt that spatial relationship, is thereby relinquished and the living substance disintegrates and dies.

Bearing in mind these several considerations, we may now give attention more especially to the results obtained in centrifuging the immature eggs of *Urechis caupo*. It will be recalled that throughout these experiments the strength of force applied was approximately 4,800 times gravity, but that the period of its duration varied from 1 hour to 18 hours. This magnitude of centrifugal force is somewhat less than three-fourths of that (6500×) used by Lyon (1907) on *Arbacia* eggs, but the time of duration is probably several times greater than any hitherto recorded. This extraordinary endurance of these *Urechis* eggs, as formerly noted, was largely due to their very tough membrane.

The number of lots of eggs centrifuged for each hour-period of from 1 to 12 hours varied from two to six. Only one lot was run for the 18-hour period. The total number of eggs centrifuged, the relative positions of whose polar bodies were carefully recorded, was 14,045.

It will be remembered that the five relative positions of polar bodies on the centrifuged eggs were conveniently assigned as fol-

lows: (1) red pole, (2) hyaline hemisphere, (3) equator, (4) opaque hemisphere, and (5) black pole. Table II gives the serial number of each lot of eggs centrifuged and lists the relative number of eggs with polar bodies occurring in each of these five positions, and their percentages respectively for each lot. In two other columns, it lists also for each lot of eggs the sum of the number of eggs having polar bodies at the red pole and on the hyaline hemisphere, and the sum of those with polar cells at the black pole and on the opaque hemisphere, together with their percentages, respectively. For general comparisons, these last-mentioned columns may be more conveniently referred to.

It will be observed from this table that there appears a very considerable overlapping of percentages, particularly in adjacent periods. It would be reasonable to expect that for the relatively crude method employed, some discrepancies should show up. Various factors might account for such: (1) variations in temperature during a given run and as between different runs; (2) differences in the relative salinity, hydrogen-ionic or the gaseous content of the sea water in the centrifuge tubes before, during, or after centrifuging; (3) differences in the age of eggs in the same female or in different females; (4) the "physiological state" or relative well-being of the female after the removal from its natural habitat and during its life under artificial conditions in the laboratory; (5) inadvertent contamination of the centrifuge tubes or other glassware; (6) variations in the revolutions per minute of the centrifuge due to fluctuations in the city current or to inadequate lubrication, etc. Such factors might help to account for a higher percentage of polar bodies (52.9 per cent) on the hyaline hemisphere (plus red pole) of the 2-hour lot No. 5 than that (51.4 per cent) of the 3-hour lot No. 9. But it is not understood why, for example, the percentage (65.2) of polar bodies on the hyaline hemisphere (and pole) of a 6-hour lot, No. 15, should have been so nearly equal to that (66.1 per cent) of the 9-hour lot No. 30, or of the 11-hour lot No. 33 (67.3 per cent).

From the comparison of percentages throughout Table II, however, the general trend of results is quite evident, viz., that as the period of duration of centrifuging is increased there is also an increase of percentage of polar bodies on the hyaline hemisphere and

the red pole. And the sum total of eggs centrifuged for each of the hour-periods (from 434 [11 hours] to 2,092 [7 hours]) is large enough, it would seem, to reduce errors in observation, recording, etc., to a reasonable minimum. It should be noted, too, that for each lot of eggs, abnormal ones ("cytolyzed," "no polar bodies present," "unfertilized," etc.), as well as those having polar bodies, were recorded. Three lots of centrifuged eggs (a 3-hour, a 9-hour, and one 12-hour lot) were discarded because, for reasons unknown, a large percentage were cytolyzed. Moreover, the 18-hour series contained, as estimated, about 10–15 per cent of cytolyzed eggs, and for this reason the percentages of distribution of these polar bodies are regarded as unsatisfactory, although they are included in Table II.

In a former paragraph of this discussion it was emphasized that any significance attaching to this general tendency of a gradual shifting of polar bodies toward the hyaline hemisphere of the centrifuged eggs of *Urechis* must depend upon whether or not the position of these polar bodies actually defines the direction of the polar axis. The evidence presented, while not complete, indicates that the orientation of polarity in these experimental eggs is in fact so defined: (1) the first and second cleavage planes pass through or near the origin of the polar cells, the first plane occasionally separating the two so that each blastomere receives a polar cell; (2) the pattern of the disposition of stratified inclusions in the immature egg apparently persists throughout the cleavage stages; (3) the trochophore larva of the centrifuged egg, in each of the 17 cases carefully followed, maintained the pattern of stratified materials with respect to the axis originally defined by the position of polar bodies in the fertilized egg; (4) the incomplete studies of sectioned and stained eggs and larvae thus far suggest that the direction of the polar axis in centrifuged *Urechis* eggs is fixed by the point of origin of the polar cells.

If the polar axis subsequent to the centrifuging of immature eggs comes to be designated in the fertilized egg by the place of extrusion of the polar bodies, and if that place of extrusion is amenable to a progressive shift that tends to vary directly with the period of duration of the applied centrifugal force, then it would appear that the orientation of polarity in the *Urechis* egg is in that respect experi-

mentally mutable. On the basis of this interpretation, to what factor or factors in the centrifuged egg may we rightly attribute this mutability?

It should be emphasized in this connection that we are not concerned here with a modification of polarity in the usual sense of that term because, as has been repeatedly pointed out, in the immature egg of *Urechis caupo* there is apparently no discernible mark of polarity and, obviously enough, what is not known to exist cannot be experimentally modified. But the results do indicate that through this magnitude of centrifugal force some condition can be imposed upon these immature eggs which limits the direction that the polar axis may assume upon becoming visibly established in the fertilized egg and that this effect increases with the duration of the applied force.

Are we to infer, then, that this condition induced by centrifuging amounts to an augmentable displacement of some inclusion or inclusions in the immature egg which produce or determine polarity in the fertilized egg? As previously stated, the consensus of opinion reached in similar studies by various investigators is that visibly stratifiable inclusions of the egg do not condition its polarity, for the axis of stratification and the polar axis do not tend to coincide. But might we not suppose that some inclusion which may be responsible for the apparently mutable orientation of polarity in the *Urechis* egg represents an invisible material—say of colloidal dimensions? The reply to this question, for the present at least, must be in the negative, for it has been shown by Svedberg (1928) that the forces required for an initial displacement of a colloid ($>$ 10,000 times gravity) are enormously greater than the force exerted on the eggs of *Urechis;* and the factor of the duration of the force is, of course, without any significance until an initial displacement is effected.

There is a further possibility, however, that this induced effect in these centrifuged eggs is of secondary origin. We may assume, for example, that some material which stratifies with difficulty because of its low specific density tends to accelerate locally the cell's metabolism or otherwise affect the cell's polarity, and so, according to Child's (1929) well-known theory, an axial gradient comes to be established. That region of higher metabolic rate, then, becomes the

region of the animal pole, and there the extrusion of polar bodies ensues.

In view of this possibility, especial attention was given to the red granules whose behavior has been described in earlier paragraphs. It was noted that these granules which come to lie in the hyaline hemisphere stratify very poorly, if ever actually. They tend rather to form irregular clusters which dispose themselves with seeming indifference, although the greater mass of these granules at times may become definitely concentrated at the centrifugal pole. No evidence was obtained, however, which would indicate that their disposition in any way influenced the place of origin of the polar bodies. Furthermore, eggs not infrequently appeared which were almost devoid of red granules; yet when such eggs were centrifuged (see Nos. 21 and 29 in Table II), the relative percentage of polar bodies on the hyaline hemisphere (and red pole) checked favorably with these percentages of other centrifuged eggs having an abundance of the red pigment.

Mention was made also of the sparse, gray substance in eggs centrifuged 7–10 hours which appears fairly distinct but indefinitely disposed toward the centrifugal pole. There was no evidence, however, that this gray material may have any influence upon the orientation of the polar axis.

Possible effects of the external medium and other environmental factors, such as the relative availability of O_2, were also taken into account during these experiments, but without success. It was decided, finally, that search for any visible inclusions or any environing conditions which might influence secondarily the direction assumed by the polar axis in this fertilized centrifuged egg must, for the present at least, be abandoned.

The remaining substance of the centrifuged egg which now invites further consideration is the hyaline protoplasm, and this appears to afford a plausible solution of our problem. Primarily it is not the shifting and stratification of visible inclusions in this egg that matters; it is rather that these inclusions displace the hyaline protoplasm. We have already emphasized the marked difference in the behavior of the black inclusions and the red granules, especially under longer periods of centrifuging. The former readily stratify and condense at the centripetal pole as a crescentic black cap, while

the latter resist stratification and tend rather to form irregular clusters or to disperse unevenly through most of the hyaline hemisphere. The behavior of the black material obtains also for the brown and the yellow inclusions—all three of which, it will be recalled, tend to form sharply defined strata within the opaque hemisphere. Here they come to displace more and more the hyaline protoplasm, which in turn becomes shifted increasingly toward the hyaline hemisphere. If polarity of the cell be regarded as a formative condition arrived at epigenetically, then in the immature *Urechis* egg we may reasonably assume that its polarity, which inheres in the hyaline protoplasm, remains nascent until the onset of processes which eventuate in the extrusion of polar bodies and the establishment of a discernible polar axis. Whether or not this nascent state involves a predisposition of polarity in the normal ovocyte, the data presented only indicate that in the centrifuged egg the range of orientation of polarity is limited through the induced limitations of the ground substance.

This interpretation accounts, then, for the increasing occurrence of polar cells on the hyaline hemisphere proportional to the relative increase of the hyaline protoplasm within that hemisphere as effected by prolonged centrifuging. It accounts, also, for the diminutive size of the few polar cells that were extruded in the region of the dense black cap where the hyaline protoplasm is most meager. It helps to explain, too, the deformed astrosphere of the cleavage spindle when that end of the amphiaster closely approaches or abuts against the dense strata of the opaque hemisphere, for the amphiaster is differentiated hyaline protoplasm. It clarifies, moreover, a striking behavior of early cleavage in these centrifuged eggs when the plane of cleavage passes through, or closely diagonal to, the axis of stratification, whereupon the constriction of the cell advances far into the active protoplasmic mass before it comes to appear in the inert strata of the opaque hemisphere. Also, it indicates how it happens that the sperm rarely, if ever actually, enters the region of the dense black cap of the centripetal pole where the essential hyaline protoplasm is quite or nearly wanting. Furthermore, it offers an explanation of the results obtained upon allowing centrifuged eggs to remain unfertilized for many hours. Meanwhile the stratified inclusions

gradually redispersed; and the hyaline ground substance, responsible for polarity and polar body formation, in its turn tended to recover more and more its location originally assumed in the uncentrifuged egg, and so the distribution of the points of extrusion of polar bodies correspondingly approached the normal.

The interpretation here presented may have an application to certain findings of Conklin (1910) in the eggs of *Lymnaea* which were centrifuged also in the germinal vesicle stage. He noted that the stratification was sharply marked and that upon maturation of a group of 25 eggs "all but 5 had polar bodies on the clear zone," i.e., the 20 pairs of polar cells were extruded, apparently, as in the centrifuged *Urechis* eggs, from the region of the hyaline protoplasm.

Certain results of the experiments of Morgan and Lyon (1907), who showed that the majority of micromeres in centrifuged *Arbacia* eggs were formed at the centrifugal or pigmented pole and that in most of the plutei the pigment was localized between the mouth and anus, seem to be fairly comparable with some of the results here reported for centrifuged Urechis eggs (see p. 442); and their findings may admit of an interpretation such as the one here proposed.

This interpretation also has a bearing upon the results of an experiment by Chambers (1928) on centrifuged sea-urchin eggs, who found that the non-granular zone of hyaline protoplasm which had been excised by a micro-needle took a membrane and underwent cleavage in the absence of the granular inclusions. Not only were the inclusions not essential to early normal development, but obviously the primary basis of that development was the hyaline ground substance.

Finally, it is clear that the point of view here adopted agrees essentially with that formulated by earlier workers (see p. 424), as reviewed in former paragraphs. It would assign to the hyaline substratum of the living cell the place and rôle of organic differentiation. It would regard polarity as a primary mark of that differentiation, emphasizing that polarity is a process as well as a condition—a formative process which is essentially epigenetic, and so a time factor is involved. Polarity then would begin with the incipient differentiation of the living egg and continue throughout development.

This point of view, furthermore, would identify the hyaline sub-

stratum or ground substance with protoplasm, a concept which, it is urged, merits today a re-emphasis when the subject of protoplasm has become so much a major interest in all biologic thought and investigations.

SUMMARY

1. The eggs of *Urechis caupo* are shed in the germinal vesicle stage and normally remain immature until fertilized.

2. The immature egg apparently has no mark of polarity. Neither the position of its one or more depressions nor the eccentricity of the germinal vesicle nor the point of entrance of the sperm could be correlated with the subsequent place of extrusion of the polar bodies.

3. In both normal and centrifuged eggs, the place of extrusion of polar bodies marks the animal pole.

4. The first cleavage plane is predictably defined by the position of the polar bodies and the point of entrance of the sperm in both normal and centrifuged eggs. Early cleavages are adequal.

5. Gastrulation occurs diametrically opposite to the position of polar bodies. This obtains in centrifuged eggs irrespective of the axis of stratification.

6. An applied centrifugal force of 4,800 times gravity stratifies the immature eggs within 3 hours into five zones which conveniently demarcate the egg into hemispheres: the centrifugal hemisphere of hyaline protoplasm with many red granules that cluster toward the outer pole; and the centripetal hemisphere, which is rendered opaque by yellow, brown, and black strata, the last forming a black cap at the inner pole. The germinal vesicle abuts against the yellow stratum, and its nucleolus is driven centrifugally against the vesicular wall or eventually through the wall into the hyaloplasm.

7. During subsequent development of the centrifuged immature eggs of *Urechis caupo:*

a) There is an increasing occurrence of polar bodies on the hyaline hemisphere proportional to the relative increase of hyaloplasm within that hemisphere as effected by prolonged centrifuging (from 1 to 12 hours);

b) Polar cells are rarely extruded in the region of the black cap where the hyaloplasm is most meager, and are markedly diminutive in size;

c) The sperm rarely, if ever, enters the egg in the region of the black cap;

d) The fertilization membrane appears first on the hyaline hemisphere and increases there more rapidly and extensively;

e) If the amphiaster abuts against the dense yellow stratum, it becomes deformed or remains unformed there;

f) When the first plane of cleavages passes through or closely diagonal to the axis of stratification, constriction of the cell advances far into the hyaline hemisphere before it appears in the opaque hemisphere;

g) If fertilization is delayed many hours after centrifuging, the stratified inclusions gradually redisperse, the hyaline ground substance tends to recover its location originally assumed in the uncentrifuged egg, and the distribution of the points of extrusion of polar bodies correspondingly approaches the normal.

The point of view adopted, which agrees essentially with that formulated by earlier workers, would identify the hyaline substratum of the living cell as protoplasm and would assign to that substratum the place and rôle of organic differentiation.

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ULTRA-VIOLET RADIATION AND THE PRODUCTION OF TWINS AND DOUBLE MONSTERS[1]

(One plate and forty-one figures)

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IN THESE studies with ultra-violet radiation on *Fundulus heteroclitus*, it was found, first, that together with twin production came the production of a series of differentially inhibited single embryos; second, that one or both members of a pair of twins or double monsters might exhibit the same type of developmental modifications as are found in single embryos; and third, that when such a condition obtains in one component of a double monster, the effect in the inhibited member is due to two factors: (*a*) an original handicap imposed by the environment before axial delimitation has taken place, and (*b*), superposed upon this, the *continued* inhibitory and dominating influence of the more normal member apparently growing at the expense of the inhibited member.

It seems best first to describe the types of abnormalities found in single monsters, and to correlate them with the various developmental periods, and then to indicate possible relations of the same sort in twins and double monsters.

In earlier studies made with *Fundulus heteroclitus*, and reported in 1925, it was found that the developing eggs were differentially susceptible to the action of sublethal doses of ultra-violet radiation. Those regions of the embryo which at the time of effective action of the radiation were most active physiologically showed the highest degree of modification in the greatest proportion of cases. Stockard (1924) made a similar observation in his studies on eye develop-

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ment. In our studies various types of developmental modifications were produced, affecting particularly the nervous and circulatory systems; early stages of development were more susceptible to a given dosage than were later stages.

Inhibition of development by exposure to radiation during early cleavage stages may persist long enough to make its effect felt in later stages of development. The fact that inhibiting influences produce their effect before the appearance of any morphological differentiation can be detected indicates an early physiological basis which determines a difference in susceptibility of the various regions of the embryo. For a more complete discussion of physiological activity and differential susceptibility to various agents, the reader is referred to studies by C. M. Child, particularly to a recent publication in *Protoplasma* (Child, 1928).

In our later studies concerning the production of abnormalities in *Fundulus* by means of ultra-violet radiation, particular attention was paid to the period of development which seemed most favorable to twin production, namely, the period just before the first cleavage. Indications of the existence of such a period were found in my earlier studies on *Fundulus* and ultra-violet radiation (Hinrichs, 1925). Stockard (1921) also points out the existence of an early limited effective period for twin production. In our later series of experiments, an effort was made to determine, as accurately as possible, the time limits of this period and to correlate the types of twins produced with the various divisions of this period. Also, it seemed important to study the early development of specific instances of double monsters. A number of such forms were isolated as early as the first indications of any axial delimitation were visible, and euscope sketches were made at intervals during subsequent development.

Since the literature on fish teratology is so voluminous, and since most of it has already been covered in reviews by Newman (1917, 1923) and by Stockard (1921), it seems best to confine references to reports of actual experimental production of twins, and to omit references to anatomical descriptions and records of twins found in embryological collections.

METHODS

In these experiments, performed at Woods Hole during the summers of 1928, 1929, and 1930, the procedure was similar to that followed in experiments reported in my earlier paper (Hinrichs, 1925).

Eggs were obtained by "stripping," and were immediately mixed with fresh sperm in a "dry bowl" to insure a high percentage of fertilization. Then the fertilized eggs were washed with sea water and, at suitable intervals thereafter, exposed to the unscreened radiation from a Cooper-Hewitt quartz mercury-vapor arc, running at 42–60 volts and 4.6–4.0 amperes on a direct-current circuit. Exposures were made in a shallow layer of sea water, just deep enough to cover the eggs, and were usually made at a distance of 11.5 cm. from the center of the arc, for a period of 1 minute, at intervals varying from 5 to 116 minutes after fertilization. (A few 2-minute exposures at greater distances were made but were found not to be as effective in twin production.) Eggs were gently stirred throughout the exposure, to prevent clumping and to insure a more uniform exposure of the lot.

The exposed eggs were again washed and separated from each other to provide adequate conditions of aëration. Development was allowed to continue at room temperature. Water was changed every 12 hours. Infertile eggs and eggs killed by radiation (distinguishable by their white opaque appearance) were removed during the first 24 hours after exposure, and the percentage of the latter recorded in column 5 of Table I, under the heading of "Early Dead." Such eggs had either been killed outright by the exposure or were able only to form membranes, but not to develop farther.

It is interesting to note, at this point, that the *Fundulus* material varied considerably during the three summers, being poorest in 1930. At no time were results used, unless the control lot of the series showed a high percentage of normal development. On this basis it was found necessary to discount experiments as follows; 2 of a total of 19 in 1928, 4 of a total of 16 in 1929, and 17 of a total of 25 in 1930. In 1930 unusually high temperatures and the temporary presence of toxic substances in the sea water were factors in reducing the number of series which could be used.

RESULTS

It will be seen that the total mortality is high, as shown in column 4 of Table I (35.0–65.4 per cent). Mortality in unradiated lots runs from 32.3 to 55.5 per cent. At intervals during development, dead eggs were removed from the dishes, and the percentages of these recorded under "Late Dead" in column 8 of Table I. Most of these latter eggs died during early cleavage or during gastrulation.

TABLE I

SUMMARIZED RESULTS OF ALL EXPERIMENTS

(1) Minutes after Fertilization	(2) No. of Exp.	(3) Total No. of Eggs	Percentage Calculations				
			Of Total Eggs			Of Total Embryos	
			(4) Total Dead	(5) Early Dead	(6) Total Embryos	(7) Normal Embryos	(8) Late Dead
5–9	5	1,080	56.3	52.6	43.7	49.8	8.5
10–14	40	9,736	54.8	45.3	45.2	49.3	21.4
15–24	28	7,993	58.2	42.9	41.8	39.3	36.7
25–35	22	6,617	62.7	56.7	37.2	58.0	16.4
40–60	8	1,333	63.1	.....	36.8	92.2	.....
75–116	7	2,873	35.0	23.7	64.9	59.4	17.3
120–150	4	1,037	65.4	.....	34.5	86.3	.....
180–340	4	2,761	55.0	.....	44.9	95.0	.....
1,120–2,080	6	2,781	58.9	.....	41.1	81.3	.....
Controls:							
1924 (summer)	17	2,656	32.3	.....	67.6	99.2	.....
1928 (summer)	17	2,690	40.7	35.2	59.2	87.2	9.0
1929 (summer)	12	2,718	55.5	54.3	44.5	95.9	2.7
1930 (summer)	8	1,740	42.1	29.9	57.9	71.5	21.1

A small group died later as the result of developmental incompetencies, as, for example, those which had a badly impaired circulation. A small percentage of such forms appear normal, except for their poor or totally lacking circulation. They may live for some time, i.e., until the residual food material of the cells of their tissues is depleted or until asphyxiation and accumulation of waste products takes place. Many instances of such embryos are reported in the literature on fish teratology.

Table I shows the number of experiments made during the various periods of development (cols. 1 and 2), the total number of eggs used in radiated and in control lots (col. 3), as well as the percentage of

eggs which formed embryos (col. 6), and those which developed normally (col. 7). It is apparent from a study of Table I that, in general, the total mortality is higher in radiated lots than in non-radiated lots, and that a larger percentage of eggs formed embryos and that of these more developed normally in the non-radiated control lots than in radiated lots. In the radiated lots the abnormalities, as previously indicated, appeared for the most part as disturbances of the circulatory and nervous systems. In non-radiated lots the small

TABLE II

PERCENTAGES OF VARIOUS TYPES OF ABNORMALITIES PRODUCED DURING THE VARIOUS PERIODS OF DEVELOPMENT

(1)	(2)	(3)	PERCENTAGE CALCULATIONS				
			(4)	(5)	(6)	(7)	(8)
MINUTES AFTER FERTILIZATION	NO. OF EXP.	TOTAL NO. OF ABNORMAL EGGS	Abnormal Embryos from Total No. of Embryos	Abnormal Circulatory System	Abnormal Eye Development	Abnormal Axial Relations	Twin Formation
5– 9.....	5	197	41.7	67.0	17.2	14.2	1.5
10–14.....	40	1,281	29.3	35.0	24.1	33.6	7.2
15–24.....	28	757	24.0	51.2	22.2	22.5	4.0
25–35.....	22	625	25.6	34.7	20.3	36.0	8.9
40–60.....	8	36	7.8	30.6	41.7	25.0	2.7
75–116.....	7	434	23.3	81.5	6.2	10.8	1.4
120–150.....	4	50	13.7	40.0	32.0	24.0	4.0
180–340.....	4	62	5.0	29.0	53.2	17.7	0.0
1,120–2,080.....	6	206	18.7	17.0	39.3	43.7	0.0
Controls:							
1924 (summer).	17	14	0.8	57.1	28.6	14.3	0.0
1928 (summer).	17	61	3.9	77.0	6.6	16.4	0.0
1929 (summer).	12	16	1.4	68.8	0.0	31.2	0.0
1930 (summer).	8	74	7.3	100.0	0.0	0.0	0.0

percentage of abnormalities appeared as malformations of the circulatory system and as a few instances of modifications of the central nervous system.

Data in Tables I and II are derived from experiments made during four summers at Woods Hole, namely, 1924, 1928, 1929, and 1930; while Table III includes only the work of the past three summers for which the data on twinning is more complete, and covers only the periods of from 5 to 116 minutes after fertilization.

Table II shows the number of total abnormalities, as well as the

percentage of each type of modification produced. Apparently exposures made earlier than 25 minutes after fertilization result in from 50 to 60 per cent abnormalities. (See Table I.) The percentage of abnormalities ranges from 24.0 to 41.7 per cent for all exposures made prior to the first cleavage, and from 13.7 to 23.3 per cent for exposures made prior to the second cleavage. A rhythmic susceptibility of developing eggs to various physical and chemical agents has been previously indicated for other species by numerous investigators, who found that, in general, periods of low susceptibility correspond to post-divisional stages, at which time the eggs are relatively passive. Also, there are proportionally fewer abnormalities produced by exposures made during later cleavage stages.

In Table II, column 1 shows the period after fertilization at which exposures were made, and column 2 shows the number of experiments made during each period. Column 3 shows the total number of abnormal eggs; the percentage of these as referred to the total number which formed embryos appears in column 4. Columns 5–8 show the percentages of abnormalities classified as abnormalities of the circulatory system, of eye development, of axial relations, and of twin formation.

It will be seen, from a study of Tables II and III, that the greatest percentage of abnormalities due to early exposures appeared in the circulatory system. All exposures recorded in Table III were made before the first cleavage. The four divisions, A, B, C, and D, represent respectively the results as recorded in number and percentage for modifications of circulation, eyes, antero-posterior axis, and twin production.

In the following discussion of the various types of abnormality produced, the data recorded in Tables II and III will be considered at the same time.

A. *Circulatory defects.*—As previously indicated in column 5 of Table II, circulatory defects amounted to from 17.0 to 81.5 per cent of the total abnormalities produced during each period of exposure (McClendon, 1912) found a similarly high susceptibility of the developing circulatory system. For the period of 5–116 minutes after fertilization (Table III), this result is far more striking. Exposures made during the first 10 minutes after fertilization produce the great-

est percentage of "high pericardium" in any group (67.4 per cent). (See col. 3, Table III.) During the three successive 10-minute in-

TABLE III

RELATION OF TYPES OF ABNORMALITIES TO PERIODS AFTER FERTILIZATION

(1)	Period after Fertilization (in Minutes)									
	5–9		10–19		20–29		30–40		45–116	
Type of Abnormality	(2) No.	(3) Per-cent-age	(4) No.	(5) Per-cent-age	(6) No.	(7) Per-cent-age	(8) No.	(9) Per-cent-age	(10) No.	(11) Per-cent-age
A. *Circulation:*										
Small, poor circulation	8	16.3	206	28.1	81	23.8	26	20.3	160	44.4
Small, abnormal, poor circulation	8	16.3	222	30.3	117	34.4	64	50.0	15	4.1
High pericardium	33	67.4	305	41.6	142	41.8	38	29.7	186	51.5
Total	49	100.0	733	100.0	340	100.0	128	100.0	361	100.0
B. *Eye modifications:*										
Median cyclopian	0	0.0	9	2.3	3	1.6	3	6.3	0	0.0
Unilateral	0	0.0	7	1.8	10	5.3	1	2.2	0	0.0
Median fused	5	55.6	155	39.7	50	26.5	23	48.9	4	50.0
Incomplete eyes	3	33.3	144	36.8	88	46.5	11	23.4	0	0.0
No eyes	1	11.1	76	19.4	38	20.1	9	19.2	4	50.0
Total	9	100.0	391	100.0	189	100.0	47	100.0	8	100.0
C. *Axial modifications:*										
Embryonic masses	0	0.0	107	32.9	44	46.8	74	69.8	0	0.0
Blood islands only	0	0.0	104	32.1	8	8.5	10	9.4	14	70.0
Bent tail	2	0.0	114	35.0	42	44.7	22	20.8	6	30.0
Total	2	100.0	325	100.0	94	100.0	106	100.0	20	100.0
D. *Twins and double monsters:*										
Two normal heads	0	0.0	5	4.8	3	9.7	2	5.0	1	59.0
One normal head	0	0.0	13	12.3	7	22.6	11	27.5	0	0.0
One normal head, one small abnormal	0	0.0	8	7.5	1	3.2	9	22.5	0	0.0
Two embryonic masses	0	0.0	9	8.5	3	9.7	7	17.5	0	0.0
Lateral fusion	0	0.0	70	66.0	16	51.6	11	27.5	1	50.0
Partially duplicated heart	0	0.0	1	0.9	1	3.2	0	0.0	0	0.0
Total	0	0.0	106	100.0	31	100.0	40	100.0	2	100.0

tervals the percentage of such malformations decreases to 29.7–41.8 per cent; but during the period of 75–150 minutes after fertilization most of the developmental modifications are again found in the

circulatory system. (See col. 5 of Table II and sec. A of Table III.) As here indicated, from 29.7 to 67.4 per cent of the circulatory defects took the form of high pericardium (Figs. 24–26) and feebly beating string hearts with little or no circulation; from 32.6 to 70.3 per cent were slightly smaller than the controls and had a sluggish circulation, and only about half of these were normal in appearance when exposures were made early in development. Many were almost entirely devoid of pigment, the development of which seems to parallel that of the circulation.

B. *Eye-defects.*—When all four summers' experiments are considered, as in Table II, it will be found that the greatest percentage of eye modifications (41.7–53.2 per cent) are seen to occur when exposures are made at from 40 to 60 and from 180 to 340 minutes after fertilization, or, in other words, in each case just following the period in which the greatest percentage of circulatory defects were produced. When only the exposures are considered which were made at or before the first cleavage, as in Table III, section B, the greatest number of eye modifications are seen to follow exposures made during the second 10 minutes after fertilization, and appear as modifications of the relation of the eyes to the surrounding tissues and to each other. Eye modifications produced by later exposures take the form of incompleteness of development or failure of realization of developmental potencies of individual eye primordia.

In cases of abnormal development of the eye region, most of which involve brain and anterior head malformations, modifications may vary in degree, as indicated in Table III, section B, from slightly approximated normal eyes to a single median eye in which indications of a double origin may or may not be present. Such forms are distinct cases of differential susceptibility, where the median anterior, and usually the dorsal region of the head, shows the greatest modification or may even be lacking. With the greater degree of approximation of eyes, and the failure of parts to develop which later lie in the interocular region, comes a simultaneous abnormality of the jaw region. The upper jaw may be entirely lacking in more extreme cases and the lower jaw assume a beaklike appearance.

In 11.1–50.0 per cent the embryos had no eyes (Table III, sec. B) and were, for the most part, undersized and with or without addi-

tional defects of circulation. A small proportion of these embryos showed independent lens formation, in place of fully developed eyes on one or both sides of the head. These lenses may or may not be normal in position, and may be extremely superficial (Fig. 1, plate I) or be buried deep in the head tissues. Approximately 20 per cent of the embryos which have eye modifications showed no lens formation and eyes appeared only as black pigment spots, usually normal in position (Figs. 21–23). Only 0.0–6.3 per cent showed median cyclopia, and 0.0–5.3 per cent developed a single lateral eye which was normal in size and position.

In studies made during 1929 and 1930 it was found that when only one eye of a pair developed, or when one eye developed more normally than the other, more often it was the right eye. For example, following exposures made during the period of 10–14 minutes after fertilization, 64.3 per cent developed more normally on the right side; while during the period of 15–24 minutes, the proportion of normal right eyes was 52.2 per cent; and during the next 10 minutes, 57.1 per cent. The foregoing calculations are based, for the first period, on 28 cases of unilateral normal development; for the second period, on 23 cases; and for the third period, on 14 cases. More often, however, during these periods, both eyes were modified in position or median cyclopia was produced.

C. *Axial modifications.*—As indicated in col. 7, Table II, such modifications comprise from 10.8 to 43.7 per cent of the total abnormalities, and result in the production of embryos which are small, twisted, and have poor circulation, and may or may not possess normal eyes, or else they may appear only as axiate embryonic masses, or even anaxiate masses, in which the integrative action of the nervous and circulatory systems have been greatly interfered with; there may even be a complete lack of embryonic tissue other than a beating heart, or isolated blood islands and pigment spots may appear alone on the yolk sac. In less extreme cases, only the development of the tail may have been interfered with, the rest of the embryo being apparently normal. Such forms may have short, stubby tails (motile or non-motile); or the tail may be normal in size and be bent at an angle to either side (Figs. 15, 22, 46–47) or dorso-ventrally and may be partially or wholly rigid (Fig. 10, Plate I). None of the

PLATE I

FIGS. 1–10.—Photomicrographs of mature embryos of *Fundulus heteroclitus*. All but one pair of twins, shown in Figure 10, were able to hatch.

FIG. 1.—Double monster in which the right eye is normal in size and position, the median eye has an extra lens superficially placed on the anterior surface of the head, and the left eye is paired. Doubling has occurred in the anterior region of the head.

FIG. 2.—Two completely separate normal individuals.

FIGS. 3 AND 4.—High- and low-power views of a double monster in which the head is partially double. The left eye is double in origin, resulting from a fusion of the right eye of the left component with the left eye of the right component. The right eye of the right component is normal; the left eye of the left component is absent.

FIG. 5.—Double monster in which one member of the pair is normal, and the smaller member appears only as a poorly differentiated mass on the ventral side of the yolk sac.

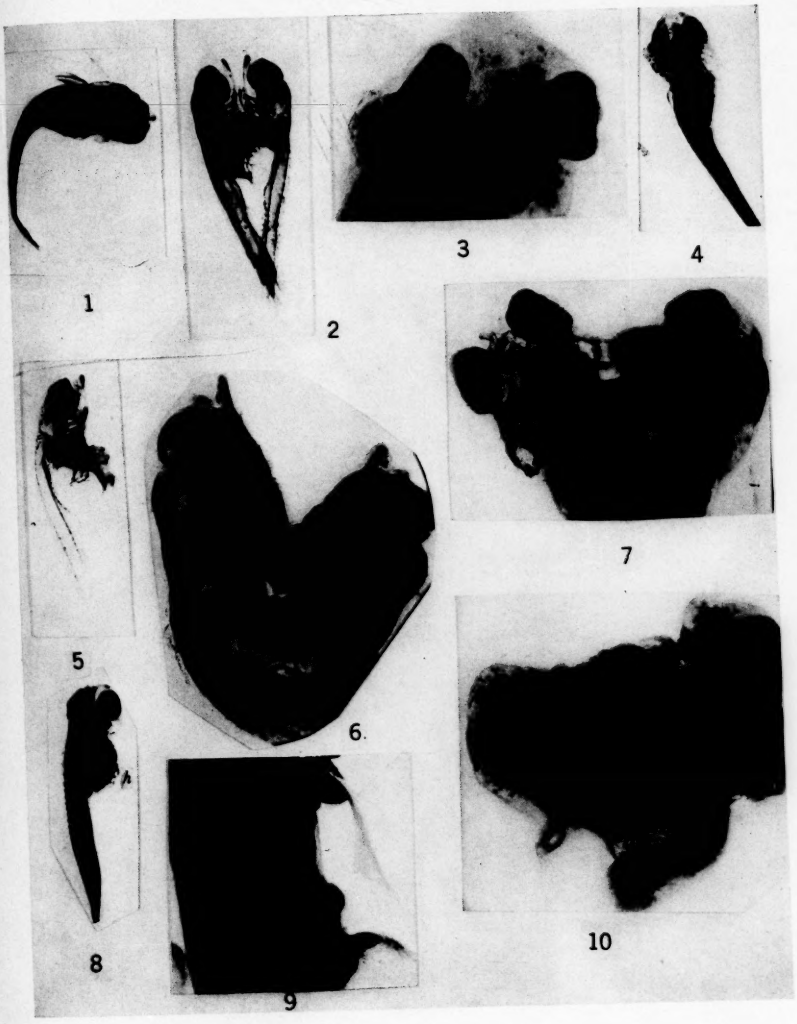
FIGS. 6 AND 7.—Double-headed monsters with both heads normal. Figure 6, embryos fused ventrally. Figure 7, embryos fused laterally.

FIG. 8.—Double monster consisting of a normal component and an extra heart (*h*), lying free on the yolk sac. The beat of the separate heart had a different rhythm from that of the heart of the normal component.

FIG. 9.—High-power photomicrograph of part of the body of an autosite-parasite monster. The parasite member appears as a small mass (*p*) on the ventral side of the body of the normal, or autosite, member.

FIG. 10.—Pair of entirely separated abnormal embryos.

PLATE I

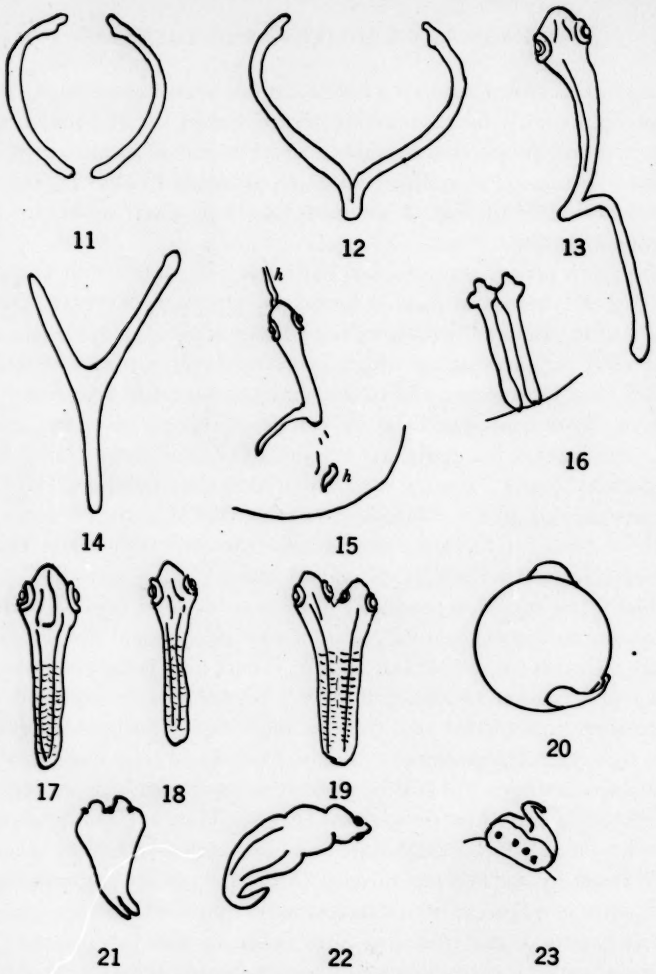


forms in this group can hatch unless the tail movements are vigorous enough to enable the embryo to wriggle out of the egg membrane. The greatest proportion of such malformations is produced by exposures made 25–35 minutes after fertilization (Table II, col. 7), which is also the time most favorable for the production of twins and double monsters.

D. *Twin production.*—So far, little has been said about the production of twins and double monsters, although the experiments were made primarily with a view of discovering, if possible, the period after fertilization at which exposure to ultra-violet radiation favors twin production, and of studying the nature of the process involved. As indicated in Table II, column 7, this period comes before the first cleavage is completed. While a significant proportion of double forms appears following radiation within the second and third 10-minute periods after fertilization, one may say that the effective period for twin production comes within the 30 minutes just before the appearance of the first cleavage furrow.

The types of twins produced have been divided into six groups according to the degree of separation and independent development of the two axes (see Table III, sec. D). There may be two completely developed normal heads, each with a separate body and yolk-sac circulation, and placed 180° from its mate and entirely independent of it throughout development (Fig. 2, Plate I). Again, both may exhibit abnormalities, yet still be entirely independent, and lie 180°, or less than 180°, apart on the yolk sac (Fig. 10, Plate I; Figs. 27, 48, 49). Or, two normal forms may start out independently and be secondarily fused in the tail region only (Figs. 11–13). In a great many cases only one head of a pair developed normally, the other appearing as a more or less inhibited form (Figs. 14 and 31), resulting, in the more extreme cases, in a small, poorly differentiated mass scarcely recognizable as an embryo, and appearing either at the lateral or ventral side of a normal embryo, and fused more or less completely therewith (Figs. 3–5, 9, Plate I; and Fig. 30). The second component may be represented only by a separate heart, beating independently on the yolk sac (Fig. 8, Plate I, and Fig. 15).

The hearts of the two components usually do not beat in unison or at exactly the same rate at laboratory temperatures where ob-



FIGS. 11–23.—Free-hand sketches of early axial relations in various types of twins and double monsters, and of double embryos which were unable to hatch and had to be removed from the shell for study.

FIGS. 11 AND 16.—Two entirely separate axes. Figure 11 shows a pair of axes which later fused in the tail region, as shown in Figure 12. Figure 16 shows a pair of axes in which fusion resulted in the formation of a double monster such as shown in Figures 17–19.

FIGS. 12–13.—Two axes connected only in the tail region.

FIG. 14.—Two axes connected more anteriorly.

FIG. 15.—Double monster in which the larger member has a bent, rigid tail and the second member is represented only by a separate heart beating on the yolk sac.

FIG. 16.—Laterally parallel axes which later fuse.

FIGS. 17–19.—Embryos resulting from the lateral fusion of a pair of axes lying parallel on the yolk sac.

FIG. 20.—Two entirely separate embryonic axes appearing as slightly differentiated masses.

FIGS. 21–23.—Double monsters with both components abnormal and fused laterally.

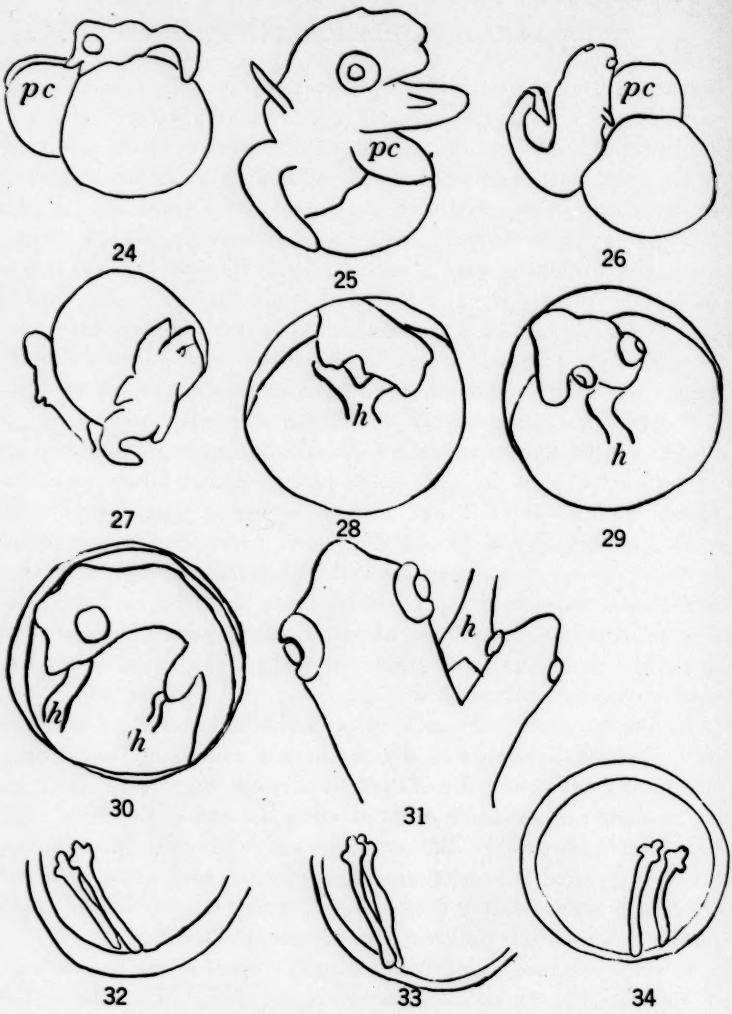
servations were at first made. Chidester (1914) records a similar observation on a single specimen. A more complete study of the relative rates of heart beat of members of such pairs of twins was made in 1929 and 1930, and will be reported in another paper. A pair of embryos may be supplied with one or two hearts (Figs. 28–31), or a single embryo may have a heart with a double origin on the yolk sac. Two such specimens were observed (Table III, sec. D), and it was found that usually the two points of origin had a different rate of beat and could be separately stimulated by ultra-violet point radiation until the time when nervous control of heart beat was established, when both points of origin were seen to beat in unison.

A significant proportion of double monsters appeared as axes in which parallel components were secondarily fused laterally in their entire length (Figs. 16, 32–34). As reorganization takes place, following such an early fusion, there is at first a partial separation visible, particularly in the anterior region, which, however, gradually disappears as a result of what looks like a melting into each other of the two components. Such embryos may have 2, 3, or 4 eyes, and a more or less ill-defined antero-posterior axis, which is wider than in normal embryos of the same age, and in which double rows of somites may be distinguished (Figs. 17–19, 35–38, 44, 45, 48, 50, 51).

By far the most common type of double monster (27.5–66.0 per cent of cases) appeared as a more or less completely fused mass which may have two pairs of eyes, or only eye spots or lenses (Figs. 21–23), and an indication of doubling in the rest of the body (Fig. 22). Varying degrees of differentiation are exhibited in these forms. Head, body, and tail region are about twice as wide as normal; and the axis is unmistakably double due to a late lateral fusion, superficially in some cases and more fundamentally in others.

In some instances, an extra median eye, itself single and normal, or obviously double in origin, may appear (Fig. 1, Plate I); or the two median eyes of a pair will fuse and one lateral one fail to develop (Figs. 3, 4).

There appears a group of embryos in which each member of the pair is greatly inhibited in development. Various degrees of inhibition are found in the separate components, such as have been described for single embryos. However, in this group the majority ap-



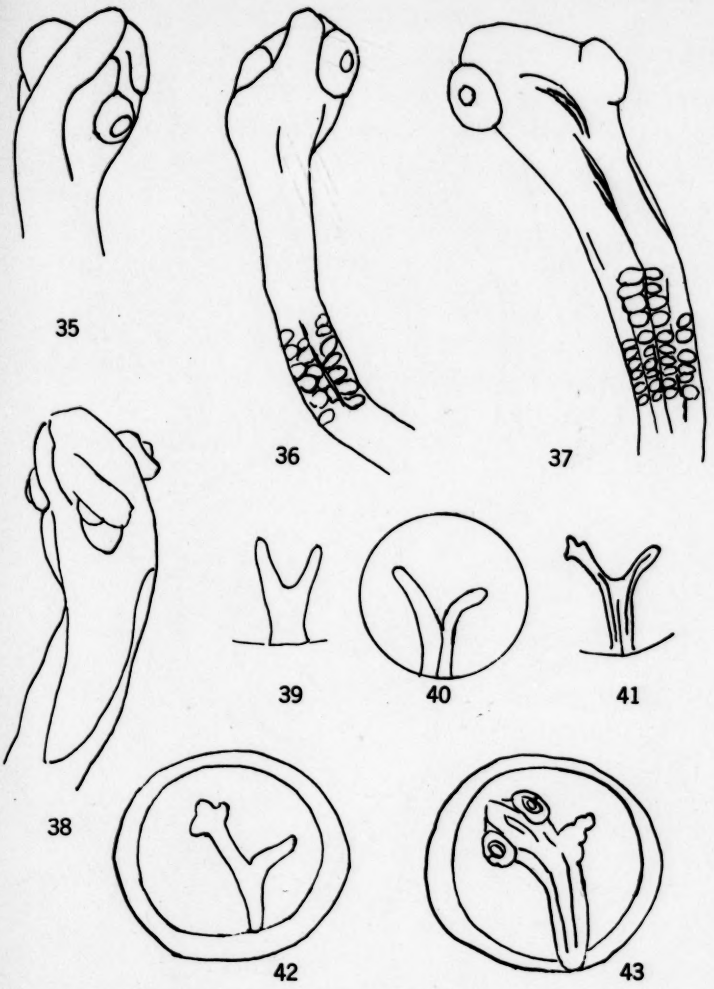
FIGS. 24–34.—Euscope sketches of single monsters, double monsters, and twins.

FIGS. 24–26.—Abnormal single embryos, showing high pericardia (*pc*).

FIG. 27.—Two entirely separate abnormal embryos on the same yolk sac.

FIGS. 28–31.—Heart relations in young double monsters. Figures 28 and 31 show a double monster with a divided heart, a single yolk-sac circulation, and a double embryonic circulation. This situation is less frequently found than that shown in Figures 29 and 30. Figure 29 shows a single heart supplying a double monster in which fusion has occurred in the head region. Figure 30 shows two hearts supplying one member of an autosite-parasite pair of twins.

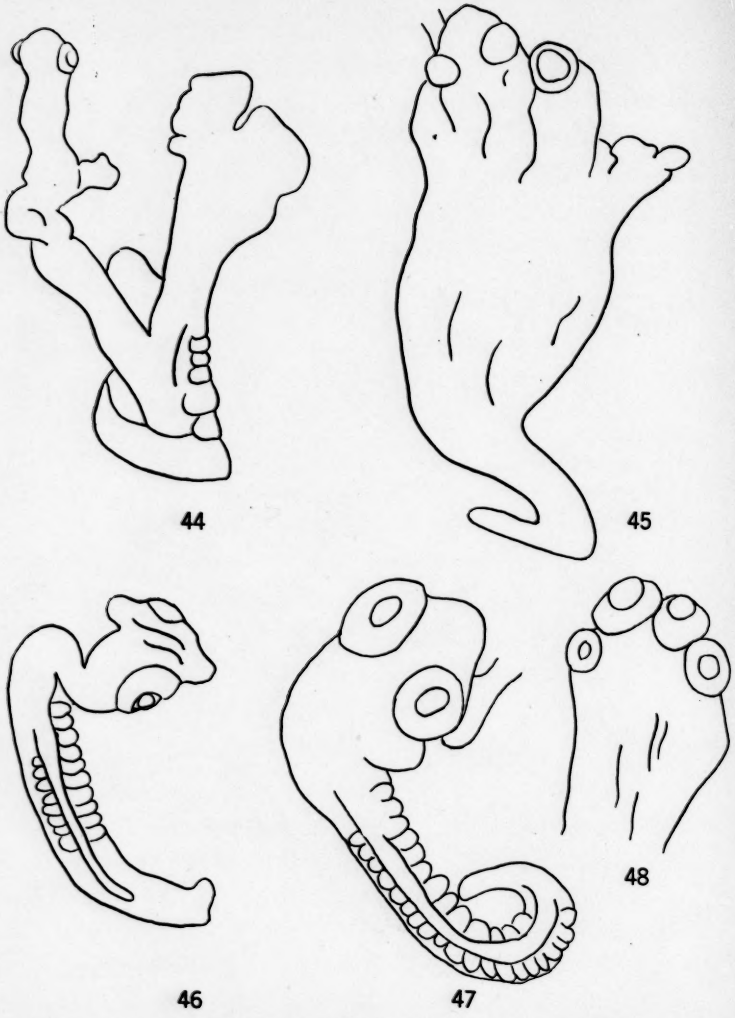
FIGS. 32–34.—Laterally paired axes, which later fused as shown in Figures 36–38.



FIGS. 35–43.—Euscope sketches of laterally fused axes, and of the historical sequence of two axes showing an unequal advantage of the one over the other, and the subsequent development of the larger component apparently at the expense of the smaller one.

FIGS. 35–38.—Irregularly fused monsters resulting from laterally paired axes shown in Figures 16 and 32–34, respectively.

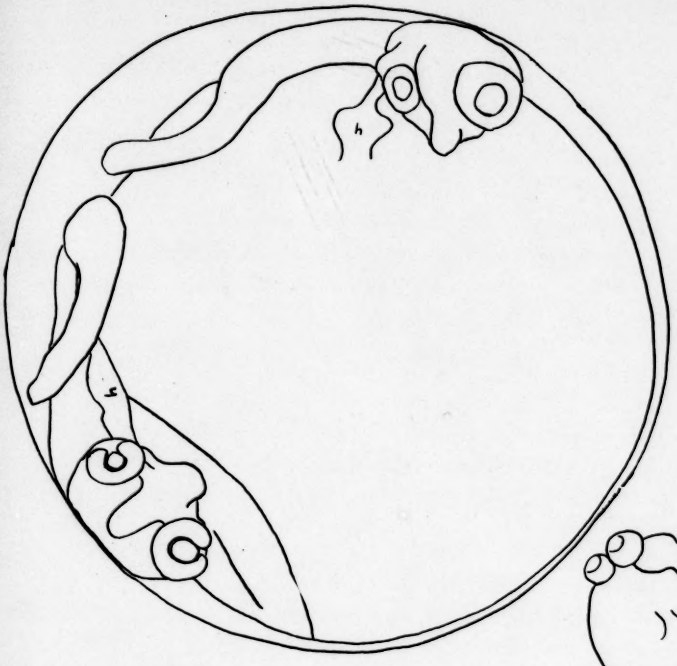
FIGS. 39–43.—A single pair of unequal fused axes, and their subsequent development. The difference in developmental rate and differentiation becomes more and more marked with age of the embryo.



FIGS. 44–48.—Euscope sketches of double monsters and laterally fused axes, and single monsters with crooked axes and bent tails.

FIGS. 44, 45, AND 48.—Double monsters resulting from the parallel development of two axes, and their subsequent lateral fusion during later development.

FIGS. 46–47.—Single monsters in which the axes are bent and the tails are rigid. Such forms never hatch.



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FIGS. 49–51.—Euscope sketches of a pair of entirely separate twins and of laterally fused double axes.

FIG. 49.—A pair of separate twins whose axes were about 150° apart on the yolk sac and which developed entirely independently of each other. The right eye of the left embryo and the left eye of the right embryo appear smaller than normal, and the points of origin of the hearts on the yolk sac are at the right side of the left embryo and at the left side of the right embryo. The axes of the embryos point toward each other posteriorly. A post-blastodermic fission of axial material is indicated.

FIGS. 50–51.—Double monsters which have arisen from secondary lateral fusions of parallel axes.

pear so completely inhibited as to consist simply of an axiate mass with little or no differentiation, and which may or may not be united with its mate (Fig. 20).

Of the total number of twins (179) produced during the summers of 1928–30, 106 were produced when exposures were made earlier than 20 minutes after fertilization. Of these, only 5 had two normal heads, 21 had one normal and one abnormal head, 9 consisted of two poorly differentiated masses, while 70 showed fusion in their entire length, and 1 pair had a partially duplicated heart.

When the various developmental periods are considered during which twin production was possible in these experiments, it will be seen that during the second 10-minute interval after fertilization, exposures of eggs to ultra-violet radiation produced the greatest percentage of parallel axial fusion, 66.0 per cent; during the next 10-minute period, 51.6 per cent of the twins were again of the same type; within the third 10-minute period, 50 per cent had one normal head; in the last period, 45–116 minutes after fertilization, exposures produced only two pairs of twins, and of these one had two normal heads, and the other was a case of parallel development of two axes, with subsequent lateral fusion, as described above.

SUMMARY

When all experiments are considered, it appears that during the first half-hour after fertilization, exposure to ultra-violet radiation produces the highest percentage of malformations in the circulatory system, especially in the heart and pericardial development. Modifications which have been classed as "axial" appear in large numbers when exposures are made 25–35 minutes after fertilization, at which time the largest group appears as axiate or anaxiate embryonic masses with little or no differentiation (see col. 7, Table II, and cols. 4 and 5, Table III, sec. C). During later exposures (45–116 minutes after fertilization), 70 per cent show a development of blood islands only, without any other embryonic differentiation visible.

Modification of development in the eye region expresses a developmental modification of the median anterior region of the developing embryo, and the periods of greatest production of such embryos come when exposures are made at from 40 to 60 minutes and at from 180 to 340 minutes after fertilization (see col. 6, Table II).

Twin production is most common following exposures made at 10–35 minutes after fertilization (Table II, col. 8, and Table III, sec. D.) In the entire four summers' experiments, it has been impossible to produce twin formation when exposures were made later than 150 minutes after fertilization.

In a few words, then, it appears that during these experiments, early exposures (at 5–24 minutes after fertilization) inhibit the development of the heart and circulatory systems most frequently; that slightly later exposures (10–35 minutes after fertilization) produce modifications in the development of antero-posterior axial alignment; and that still later (40–60 minutes after fertilization) the anterior region of the embryo is highly susceptible, as the axis had already been delimited, and its anterior end seems to be developing rapidly. Modifications of development at this period find expression in inhibitions of the forebrain and interocular regions, and approximation of eyes; at the same period, a large number of embryos appears as small abnormal forms with poor circulation and often a high pericardium, and, in many instances, with no circulation whatever. The embryos in this group are small, undernourished; and differentiation along the antero-posterior axis is less regular than during other developmental periods. A second such series of "high points" in developmental susceptibility follows after the second cleavage. Again the developing circulatory system is most highly susceptible at 75–150 minutes after fertilization. Eye development is most interfered with at 180–340 minutes after fertilization; and, following that, axial relations are again interfered with in large proportions after 1,120 minutes after fertilization (see Table II, cols. 5–8).

THEORETICAL

As has been previously stated, the modifications which appear in both single and double embryos following exposure of eggs of *Fundulus heteroclitus* to ultra-violet radiation shortly after fertilization are such as can be ascribed to a differential susceptibility at the time of exposure, or at least at the time of effective action of the radiation. The embryos produced are similar to those described by other authors and attributed by them to the deleterious action of various physical and chemical agents, or even to hybridization.

Since the exposures in these experiments were made before any

visible morphological differentiation of organs had appeared—and a great many, even before the first cleavage was indicated—there appears either to have been a preprimordial difference in the susceptibility of the egg protoplasm at the time of exposure or a latent period of activity of radiation effect, or both. There is some reason for believing that, although an agent may be applied during an early stage of development, as in some of the foregoing experiments, it may not exert an immediately noticeable influence but may become effective at a later time, when the organism reaches a more susceptible state. Experiments made with alkaloids (Hinrichs, 1929) point to a similar relatively non-susceptible period in early cleavage, with a high degree of susceptibility during gastrulation. It has been noted above that certain regions of the embryo are more susceptible to modification of development than others during a given period of embryonic development. The heart and circulatory systems present the greatest percentage of abnormalities following exposures made during early development. This would suggest that the early elaboration of embryonic and extra-embryonic tissue concerns itself with the satisfaction of food requirements of the developing organism. The developing axis is indicated by the developing nervous system; and early modification of this region finds later expression in malformed brain and sense organs, and in abnormal axial relations, such that short, stubby embryos with bent, functionless tails develop in some instances, or partial or complete double axes appear as a result of inhibitory influences effective during the period before axial delimitation begins, namely, during early cleavage. Twinning appears to be the result of a differential inhibition produced at, or just prior to, the time of delimitation of the main body axis. As indicated in Table II, columns 7–8, the greatest percentage of twin formation occurs at times when the axial relations in the embryo are easily disturbed. Twin formation and axial malformation parallel each other throughout, during the first two and one-half hours.

Stockard (1921) states that "it is probably more important to obtain the reduction in rate [of development] at an exact and very limited moment than to have a definite degree of reduction." Ultraviolet radiation, by virtue of its rapidly effective action, is a valuable agent in twin production, in this species.

It is a significant fact that early exposures produce only anterior dichotomy (anadidymy), while double-tailed forms or posterior dichotomy (katadidymy) appear only as a result of later exposures, i.e., at a time when the posterior region of the embryo is being delimited (Hinrichs, 1925). Since anterior regions are the first to develop under normal conditions, it is obvious that early exposures would interfere with development in these regions, while later exposures would alter the developmental capacity of the more posterior regions.

There are numerous theories to account for the various kinds of twins produced, and we are here concerned only with identical or one-egg twins and double monsters. It appears, from these studies, that the formation of two axes is the result of an inhibition of the "growing-tip" region of the blastoderm and that the simultaneous development of two axes is "favored when normal developmental conditions return" (Newman, 1923). The mechanism by which this is brought about is more difficult to understand, especially since exposures which are effective in twin production must come at such an early period of development and last for only a short period of time. The effective period, as previously stated, comes before the first cleavage; and the most effective period is the 30-minute interval just before cleavage is indicated. It may appear, then, that a physiological isolation of the first two blastomeres is responsible. It has been pointed out by a number of investigators that each of the first two blastomeres in many species is potentially capable of developing into a complete animal if the blastomeres are completely separated from each other experimentally. Whether the actual removal of one blastomere, by section or by ligation, can be interpreted as analogous to a situation such as exists when two blastomeres are maintained in actual physical contact but have developed a possible physiological isolation, is a question which these experiments have raised but not answered.

Another possibility to be considered in twin production by this method is that the action of radiation involves a latent period and may become effective some little time after the exposure has been made. In consequence, it may appear that the earlier an agent is applied, the longer is the time before the susceptible period is

reached. There should then be varying degrees of action of an agent proportional, to some extent, to the length of time the agent is acting, assuming constancy of action throughout. This would seem to hold for such agents as are continually applied during early development, e.g., chemicals or low temperature; but with ultra-violet radiation, which in these experiments, is applied for only 1 minute early in development, there is a chance for a latent effect as well as for an immediate action and consequent recovery, partial or complete. This seems a likely explanation for the effects of radiation, as used in these experiments. We must not lose sight of the fact that there are considerable variations in individual susceptibility and rate of physiological activity at any given period among lots of eggs numbering a thousand or more; this makes desirable the study of these results on a percentage basis.

It is certainly true that it is possible to select from eggs in early developmental stages those which give promise of developing more than one axis. Such eggs have, in many cases, been followed through early development with periodic euscope sketches made of the progress of development of the two components (Figs. 39–43).

It appears that, with the possible exception of embryos which develop 180° apart, and which are entirely separate on the yolk sac and are entirely devoid of those visible connection between them, even in their extra-embryonic circulations, the majority of double embryos develop from a *single* blastodermic area which has divided long before any evidence of axial delimitation is visible. The first sign of any axial delimitation takes the form of a single shield whose outline indicates clearly whether the embryo is to be single or double, and, if the latter, whether both parts are equipotential or not. Later stages frequently bring about a reorganization of embryonic material as a result of the interaction of dominant regions of developing axes on each other, as, for example, in the development of "autosite parasite" twins, or of the more or less complete secondary lateral fusion of parallel components, or of the superficial fusion of tail regions of primarily completely separated embryos.

The single blastodermic origin of twins and double monsters is easy to understand in forms showing *situs inversus*, or in embryos which have partially double hearts, or in such forms as shown in

Figure 49, where mirror-imaging appears in two completely separated embryos.

However, when the first indication of embryonic development takes the form of two completely separated growing regions on the germ ring, 180° apart, and these later develop entirely independently without any visible vascular connections, except as these may later appear secondarily on the yolk sac, as the extra-embryonic region of one embryo encroaches upon the other, it appears that such forms have originated from two completely separated blastodermic areas. Such cases were relatively rare in the experiments herein reported.

It was found that the closer the developing axes were to each other, the greater the chance for interaction between them. Also, when one axis has a developmental advantage over the other at the outset, it will grow apparently at the expense of the latter and form a typical "autosite-parasite" twin. Besides having an initial handicap in development, the less fortunate member of such a pair is continually subjected to the dominating action of the normal member of the pair, and usually becomes more and more resorbed until it may form just an excrescence on the body of the normal component. (Compare Newman's studies on *Patiria*, as reported in 1923, p. 26.)

Probably the most interesting series of twins which has been observed in these studies is the type in which a lateral fusion takes place between members of a parallel pair of axes which lie close together. During early development the axes appear not to influence each other in development; but at the time when lateral growth and differentiation appear, a lateral fusion takes place along the entire length of the axes, indicating a degree of miscibility of their protoplasm at this stage. There seems to be a melting into each other, probably not entirely due to crowding but also to a possible "biotaxis" between the two individuals. The final outcome is a widened axis, more or less irregular in appearance, and in which two double rows of somites are distinguishable. There is evidence of some slight dedifferentiation of primordia in the median regions along the approximating axes.

The idea of an early fission of primordia in human twin production followed by a secondary fusion was held as early as 1866 by Fischer, and reiterated by Newman in 1917 (p. 23). Evidence is pre-

sented in our experiments for such an explanation of twinning in *Fundulus*. Newman, in a private communication, states that "the facts here presented indicate that in fishes there are two distinct types of twin formation: (*a*) that in which twinning antedates gastrulation [axis formation], and (*b*) that in which twinning accompanies gastrulation." The first type would then give rise to two separate areas of gastrulation and two completely separated axes, while the latter would give rise to double monsters, chiefly of the anadidymous type. No evidence has been found in these experiments for the old idea that anadidymy resulted from a concrescence of the germ ring, followed by a budding of two anterior regions from a common stock, but rather points to their "origin by fission of an original axis (more or less complete), followed by secondary lateral fusions which may involve some melting together of internal structures."

CONCLUSIONS

1. Further evidence is presented for the differential susceptibility of embryonic organisms to ultra-violet radiation. The developing circulatory and nervous systems show the greatest degree of developmental modifications as a result of exposure of eggs to ultra-violet radiation during early periods of development.

2. One or both members of a pair of twins or double monsters may exhibit the same differential inhibitions as are found in single embryos.

3. The most effective period for the production of twins and double monsters comes during the 30-minute period just before the first cleavage.

4. Twins and double monsters were classified according to the degree of abnormality found in one or both members of a pair, and according to their relative distances apart on the yolk sac.

5. The majority of double forms arise from a single blastodermic origin. There may be secondary fusions resulting from the proximity of developing axes on the yolk sac.

6. The degree of secondary fusion seems to be a function of the original location of the growing axes with respect to each other. The farther apart they are at the outset, the better the chance of

maintaining their own individuality in subsequent growth and differentiation.

7. Because of the short period of exposure required to produce developmental modifications, ultra-violet radiation has proved itself to be an effective agent in the production of twins and double monsters in *Fundulus heteroclitus*. The optimum period of exposure is short enough to be effective during the short developmental period which is most favorable for the production of twins and double monsters.

We wish here to express our appreciation to Dr. R. S. Lillie, of the Department of Physiology, University of Chicago, for suggestions and criticism during the study of this problem, and to Dr. H. H. Newman, of the Department of Zoölogy, for critical reading of the manuscript.

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THE FORMATION OF PIGEON "MILK"

(Two plates)

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IT HAS become generally known since the observations of John Hunter (1786) that both male and female pigeons feed the newly hatched squabs on a substance regurgitated from their crops. The material which is derived from the parental crop tissue has been called pigeon "milk" (*Kropfmilch*) and has been described in textbooks of biology as constituting a true secretion. This more or less general conception of pigeon-"milk" formation naturally presupposes, according to the opinion of Neergaard and Howe and Leydig, the existence in the crop of true gland cells which secrete the pigeon "milk." The researches of Charbonnel-Salle and M. Phisalix (1886), Teichmann (1889), and Litwer (1926) have demonstrated that the formation of pigeon "milk" cannot be considered as a true secretory process. These investigators have further disclosed the fact that the formation of the pigeon "milk" is confined to two laterally situated lobes in the crop of both parent birds. The lobes undergo marked hypertrophy concomitant with the incubation period of the reproductive cycle.

It is the purpose of this paper to describe in a somewhat detailed manner the structural changes occurring in the crop during hypertrophy, formation of pigeon "milk," and the involution of the crop after hatching. Also, we wish to indicate some of the physiological problems which might be related to, or associated with, the causation of this interesting process.

MATERIAL AND METHODS

The material for this study consists of the common pigeon, *Columbia livia*. Pieces of the lobes of the crop of both sexes in a clutch were taken, as far as possible, from the same birds at known periods during incubation and after hatching. The advantage offered by this

method is the decrease in amount of variation which is found in the rate of crop hypertrophy in different pigeons of the same sex. However, it is not always possible by this method to obtain material from the same birds throughout their entire reproductive cycle. Too frequent operation on the lobes of the crop often causes an excess of scar tissue to form in the area of the incision, which is likely to interfere with the normal process of crop hypertrophy. Also, too frequent handling of the birds increases the percentages of unsuccessful clutches due to the birds more often neglecting incubation.

The hypertrophy of the crop is a continuous process, and therefore no definite stages can be sharply set off for histological study. However, for purposes of description we have collected material from the same bird as follows: before the eggs were laid and 8, 12, 15, and 18 days after the eggs were laid. To complete the cycle, material was taken from other birds, as will be indicated. In addition to this material, pigeon "milk" from the crop of the parents and from the crop of the young squab was studied.

In our experience it was found advantageous to place the samples taken from the lobes of the crop on a small piece of cardboard before immersion in the fixative (Bouin's), thus preventing the curling of the edges of the tissue and facilitating a much easier interpretation of the histological pictures. The sections were stained in Delafield's hematoxylin and eosin, Mallory's triple stain, and Mayer's hematoxylin. Regaud's method proved the best for mitochondria. For gross study, the entire crop was dissected out and placed in alcohol.

DESCRIPTION

Crop of an unmated bird.—The lobes in question are located in a lateral and posterior position in the crop. Their free epithelial surfaces bound the lumen of the crop and are consequently in direct contact with the food material. In an unmated bird the lobes are only delimited from the rest of the crop by a very slight folding (Fig. 1). In sections the crop presents a layer of stratified squamous epithelium of approximately 5–10 cells thick (Fig. 6). It is this layer of cells that becomes thrown into large folds during incubation of the eggs and is subsequently desquamated into the lumen of the crop to furnish the nutritive material of the pigeon "milk."

The epithelium of the lobes of the crop can be divided into two fairly distinct layers. The outer or superficial layer, because of its later function in the formation of pigeon "milk," may be termed "nutritive" epithelium. The deeper layer of cells, those in the region of the basement membrane, has been designated by Litwer as "proliferating" epithelium, because it is in this layer that the new cells are formed and gradually pushed outward until they take up a position in the "nutritive" layer. These two layers of the epithelium are not as distinct in the unmated bird's crop as in the hypertrophied crop. Under the "proliferating" epithelium is found the tunica propria, subjacent to which occurs a layer of transverse and longitudinal muscles. Binding the musculature and epithelial tissues together are considerable amounts of connective tissue, fairly well supplied with blood. There is, of course, as on any free epithelial surface, a certain amount of continuous cell desquamation.

Crop of 8 days' incubation.—It has been our experience to find the first notable hypertrophy of the lobes of the crop about the eighth day of incubation of the eggs (Fig. 7). The most pronounced histological changes in our series consist of frequent mitotic figures in the layer of "proliferating" epithelium and a demonstrable increase in blood supply. As a result of the frequent mitoses, the thickness of the epithelium has become correspondingly increased and thrown into distinct folds.

Crop of 12 days' incubation.—The lobes of the crop of a bird that has been incubating eggs for 12 days shows a marked hypertrophy (Fig. 8). Macroscopically, the hypertrophy of the lobes of the crop can be determined by an increase in thickness of the walls and by an increased blood supply.

Microscopic examination reveals frequent mitotic figures in the basal layers of the epithelium. The increased number of cell divisions in this region exerts a pressure upon the surrounding cells, causing considerable variation in cell form. The superficial layer of cells tends to be more or less flattened, while the deeper cells, those forming the basal layer of the stratified epithelium, are cuboidal in shape.

In the nuclei of the cells of the basal layer are found one or two karyosomes, comparatively small in size and more or less rounded

or elongated in the direction of the long axis of the cell body. In the outer layer of epithelium, where the pressure seems to be less, the karyosomes and nuclei in general appear to be somewhat larger.

Fifteen days' incubation.—At this period of incubation, the lobes of the crop can be palpated with considerable precision. The blood supply is greatly increased, and the folds of the epithelium are well under way toward fusion (Fig. 9). Fusion is accomplished by an increase in thickness of the folds produced by rapid division of the cells in the "proliferating" layer, thus increasing the width of the epithelium and correspondingly decreasing the space between the folds. Fusion may take place anywhere along the adjoining surfaces at the point of contact of the two folds. By this time the connective tissue areas in the centers of the folds, that is, the area between the two parallel adjacent basal layers, has become greatly reduced in size as a result of the pressure developed by the great hypertrophy of the epithelium. Fusion, however, never occurs between the two adjacent parallel surfaces of the basal layers of "proliferating" epithelium; consequently, connective tissue and blood vessels are always found between them.

Eighteen days' incubation.—At about 18 days' incubation (time of hatching), the lobes of the crop are approximately at their greatest development and occupy the major part of the crop (Fig. 2). They can be easily palpated; and, upon dividing the skin overlying them, one is immediately impressed by the marked increase in blood supply. If the crop is opened at this stage, considerable amounts of desquamated material are usually found in the lumen. Microscopically, the epithelium is strikingly marked off into two definite zones (Fig. 10). The "proliferating" epithelium is demarked from the "nutritive" layer by difference in staining reactions, fat content, blood supply, and cell form.

At this stage the space between the folds is completely filled by "nutritive" epithelium which has been derived from a proliferation of the basal epithelium of the folds. Consequently, a more or less continuous surface of pavement epithelium, several layers thick, covers the free surface of the united folds.

During the later stages of crop hypertrophy definite globules of fat appear in the cells of the "nutritive" epithelium. They seem to

PLATE I

Figure 1 was drawn by Alice Clarke Mullen; Figures 2–5 by H. J. Wakeman. All figures in Plate I reduced one-half in reproduction.

FIG. 1.—Drawing of complete crop of a female pigeon that was not incubating eggs. One side of the crop has been laid open to show the gross structure. Only very small folds mark the position of the lobes. Extending down from the esophagus are cords of glands termed by Dulzetto, *ghiandol parasalivari*. Approximately normal size.

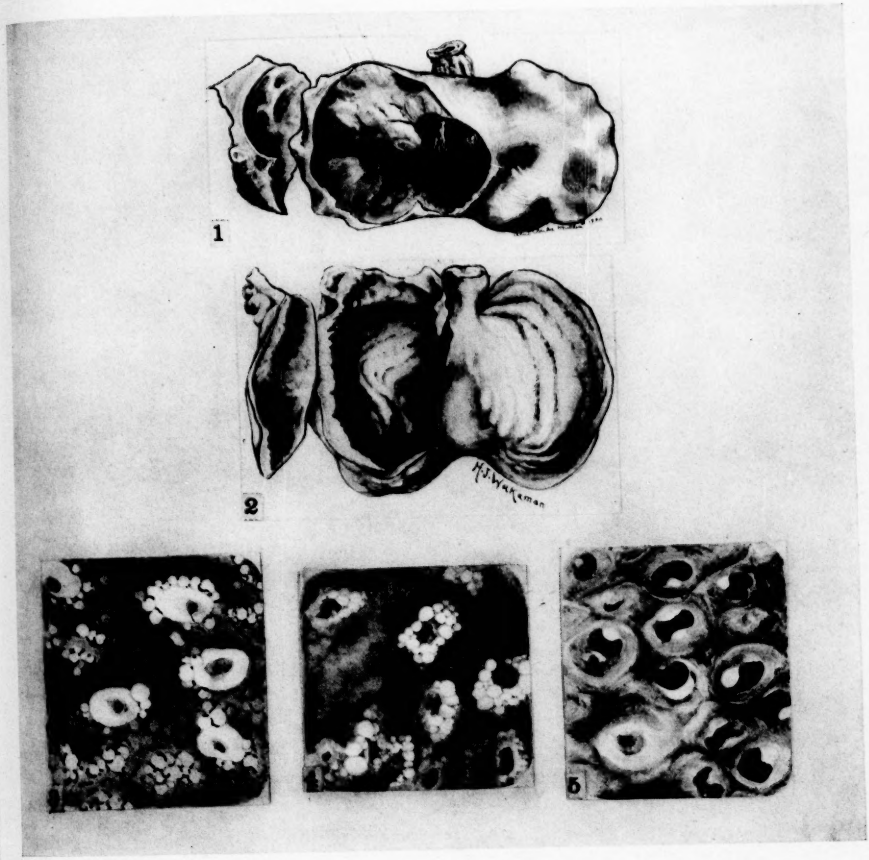
FIG. 2.—Drawing of intact female pigeon's crop 2 days after hatching of the young, with one side opened to show the marked thickness and general macroscopic appearance of the lobes of the crop. Compare with Figure 1. Approximately normal size.

FIG. 3.—Section through the hypertrophied crop epithelium of a male pigeon a few cell layers below the surface, showing the numerous rodlike mitochondria and their relation to the fat vacuoles. This section shows very little evidence of degeneration in spite of the presence of numerous fat vacuoles. Magnification approximately ×1,200.

FIG. 4.—Section of a small mass of pigeon "milk" taken from the lumen of the parent-bird's crop. Definite signs of degeneration are obvious. The vacuoles are larger and less distinct than they were before desquamation. The nuclei are shrunken, the chromatin material is clumped, and the cytoplasm appears to be undergoing autolysis. Magnification approximately ×1,200.

FIG. 5.—Section of a small mass of pigeon "milk" recovered from the crop of the young squab after being transferred from the parent crop by the feeding act. Marked signs of degeneration are present, such as shrinkage of the cytoplasm and nuclei. The vacuoles have broken down, and the tissue has undergone considerable putrefaction, autolysis, and disintegration. A pronounced change has occurred in the staining reaction. Magnification approximately ×1,200.

PLATE I



appear at about 12–15 days of incubation and continue to grow in size as the cells are pushed outward. These globules of fat make their first appearance in the cytoplasm in the immediate vicinity of the nucleus. In this connection a rather close topographical relationship exists between the vacuoles and the chondriosomes (Fig. 3). However, there was little evidence that the mitochondria pass bodily into the formation of fat. If they play a part in fat synthesis in this particular case, it would seem to be in the form of a definite synthesization (electosome theory of Regaud). Litwer found no evidence in his studies which indicates that the mitochondria are directly associated with the formation of fat. He did, however, observe a fairly close spatial relationship between the vacuoles and the Golgi apparatus.

During the hypertrophy of the lobes of the crop the outermost layers of cells are continuously desquamated into the lumen (Fig. 11). Some of the cells are more or less intact, while others show degenerative changes. As hypertrophy progresses, the cells become laden with fat. They are deprived of their blood supply and are subsequently desquamated into the lumen of the crop.

Desquamated tissue in the lumen of the parental crop (pigeon "milk").—On examining the contents of the parents' crops shortly after the young have hatched and before they have regurgitated the contents of the crops to the beak of the young squab, one finds a rather cheeselike, oily, milk-colored substance which contains in it small particles or masses of varying sizes. The milkiness is probably due to the disintegration of the fatty globules in the desquamated cells. Among this material may also be found a limited amount of food, although in some cases the entire contents of the parents' crops seem to be "milk." This is particularly true in the first few days of feeding.

On sectioning the pigeon "milk" (Fig. 4) from the lumen of the parents' crops, one finds definite cellular structures of varying degrees of organization, depending somewhat on the length of time it has been undergoing degeneration. Here can be noted the pycnotic character of the nuclei as determined by the clumping of the chromatin material, and the irregular and shriveled appearance of the

PLATE II

All figures in Plate II reduced two-thirds in reproduction.

FIG. 6.—Section through a portion of the lobe of the crop of a female pigeon that was not incubating eggs, removed by operation to show the histological structures. The lobes of the crop are composed of stratified squamous epithelium, the outer layers of which tend to be more flattened and constitute the "nutritive" epithelium. The deeper layer of cells, those which rest on the basement membrane, are irregular in shape and constitute the "proliferating" epithelium. Below the epithelium is found the tunica propria and the longitudinal and transverse muscle fibers. Magnification approximately ×600.

FIG. 7.—Portion of a lobe of the same bird, removed by operation 8 days after the bird had begun incubating eggs. The different cell layers are distinct, and an increase in thickness of the epithelium is apparent. Large blood vessels are seen penetrating the tunica propria. Magnification approximately ×600.

FIG. 8.—Section through a similar portion of the same crop, removed by operation 12 days after beginning of incubation of eggs. The increase in thickness of the epithelium is very marked, and the increased size of blood vessels is striking. Frequent mitoses of the proliferating epithelial cells are seen at this stage. Magnification approximately ×600.

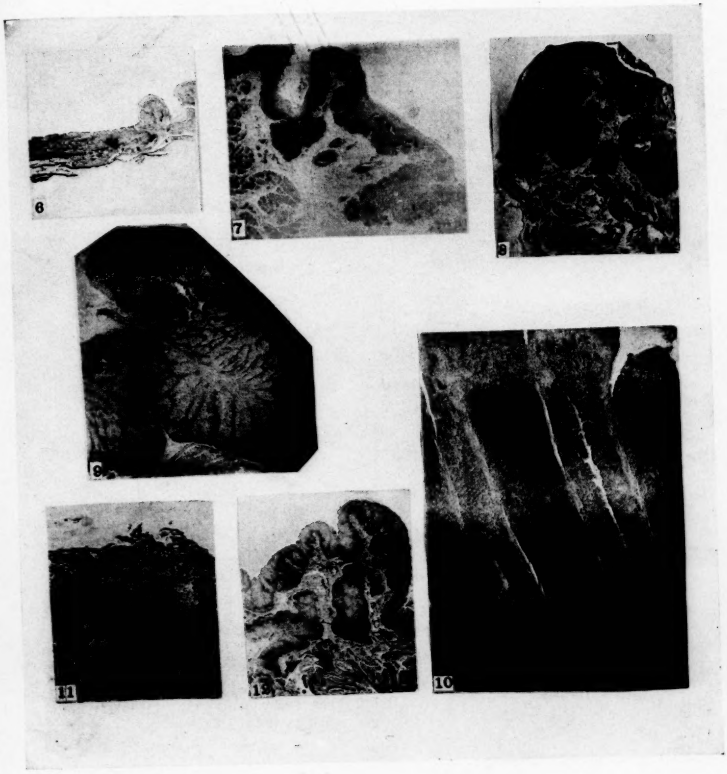
FIG. 9.—Section of a portion of the same crop as in Figures 6, 7, and 8, removed by operation 15 days after the last egg was laid. The hypertrophy has resulted in considerable increase in thickness of the epithelium. The space between the adjacent folds is almost completely obliterated. However, the area between the adjacent basal layers of the folds of the epithelium is greatly reduced, but the basal layers do not fuse and the space between them still contains connective tissue and blood vessels. The underlying muscles appear to be hypertrophied. Magnification approximately ×600.

FIG. 10.—Section through the lobe of a female pigeon's crop at the time of hatching. The two layers of the epithelium are definitely demarked as indicated by cell form, staining reaction, and possession of fat vacuoles. The mass of "nutritive" epithelium between the adjacent basal layers of the folds has completely fused and appears to be almost void of blood supply. The "proliferating" layer of cells, on the other hand, is seen to contain a good blood supply and to be relatively free of fat vacuoles. Increased thickness of the crop at this stage in comparison with Figure 1 is found to be 25–30 times greater. Magnification approximately ×400.

FIG. 11.—Superficial portion of a lobe of a female pigeon's crop during the formation of pigeon "milk." The outer cells, laden with fat vacuoles, are observed in the process of desquamation into the lumen of the crop. The cells in many cases appear to have retained their more or less definite form. Magnification approximately ×800.

FIG. 12.—This section was taken through a portion of the lobe of a female bird's crop 14 days after the young had hatched. Signs of regression are indicated by the marked decrease in thickness of the epithelial layers. Magnification approximately ×600.

PLATE II



nuclei. Some of the vacuoles are still apparent, although signs of their breaking down and dissolution are obvious.

Upon opening the crop of a bird in which much desquamated material is present, one is immediately impressed by the characteristic odor of decomposition. This decomposition is probably produced by bacteria and by enzymes which are present in the crop. These enzymes are produced by glands (*ghiandol parasalivari*) in the lower region of the esophagus said to be homologous to the salivary glands of mammals (Dulzetto, 1930).

Pigeon "milk" in the crop of a recently hatched squab.—On examining the content of the crop of a recently fed squab, one finds the same milklike material that is found in the crop of the parent bird. A histological examination of the crop of the young squab demonstrates that this "milk" is not derived from the crop of the immature bird. No indication of hypertrophy, fat globules, or desquamated epithelium is apparent. The material which has been transferred from the crop of the adult bird to the young appears in sections similar to that in the crop of the parent-bird except that further degenerative changes of the desquamated material have taken place. Figure 5 represents a portion of the cheesy material found in the crop of the squab. Definite degenerative changes are obvious. The fatty vacuoles have become completely dissolved and broken down, leaving only an unstainable area in the cytoplasm which indicates their former position in the cell. The nuclei show marked degenerative changes, such as shrinking, and clumping of the chromatin material, and in some cases complete failure to stain. Pigeon "milk" varies from the above-described, more or less cellular condition to a condition of complete loss of cellular organization.

Involution of the crop after the hatching of the young birds.—The regression of the crop of the parent-bird begins at about 1 or 2 weeks after the young have hatched (Fig. 12). Cell proliferation decreases, and a continued diminution in the amount of the desquamated epithelium is apparent. The retrogressive stages of the crop are not as definite as the developmental stages. However, it seems probable that the crop does not completely reach its former quiescent condition until about 14–25 days after hatching, depending upon little-understood factors. Some of these factors may be those associated

with the occurrence of a second reproductive cycle, culminating in the laying of eggs at the end of the second week. According to Whitman (*vide* Carr, 1919), such a female loses interest in her progeny and neglects to feed them. The male, on the other hand, may continue to feed the young for a longer time. In our experience the complete involution of the crop has taken place before the second egg-laying period has appeared. Litwer found that regression of the crop was complete at about 26 days after the young were hatched.

DISCUSSION

From the foregoing description it will appear that the formation of so-called pigeon "milk" is a unique process among vertebrate animals. It is an exceptionally interesting process in that a complete sacrifice of cell structure takes place to form the nourishment for the young. In this respect it differs from the formation of milk in that the major part of milk is formed by a pinching-off of a fat globule (that has been synthesized in the cell) which may contain only a delicate cytoplasmic envelope. The cell in this case is probably capable of many such cycles. However, this process does not constitute the whole of milk secretion; in many cases the outer portion of the columnar epithelial cell lining the alveoli of the mammary gland may sacrifice the outer tip of its protoplasm to the formation of milk. Nevertheless, it is of interest to note that the secretion (synthesization) of the globules of fat in the cells of the lobes of the pigeon crop is perhaps very similar to the formation of the globule of fat in the epithelial cells of the mammary gland.

It is apparent that the formation of pigeon "milk" may be divided into two separate and distinct phases, the first of which may be termed the "pro-secretive" phase, constituting the process by which the globules of fat are stored in the cells. The second phase is the "desquamation" phase, that is, the process by which the superficial fat-laden cells are separated from the crop epithelium and come to lie in the lumen, constituting the pigeon "milk."

Concerning the formation of fat in the "nutritive" layer of cells, two possibilities present themselves. First, is the deposition of fat due to a secretory phenomena—complementary, perhaps, with the formation of fat in the mammary gland? Or on the other hand is it a

result of fatty degeneration? Our observations suggest that the deposition of lipoids in these cells is by a secretory process. The fat droplets begin to appear in the part of the cytoplasm next to the nucleus and take on the form of small globules which may be elaborated at the expense of, and partly through, the activity of the cytoplasm or cytoplasmic components. During recent years evidence has been accumulating that the mitochondria are the principal synthesizers of lipoidal material. In our preparations we find a fairly definite topographical relationship between the rodlike mitochondria and the fat vacuoles. However, this does not necessarily mean that the mitochondria are the foci of synthetic formation of fat, and no conclusive evidence can be offered at this time regarding this particular point. This is not surprising, for, in spite of the numerous theories of secretion that have developed since the time of Johannes Müller, the secretory phenomenon still remains in doubt.

It is an interesting fact that the formation of the fat droplets begins to appear in the "nutritive" epithelium a few days following the beginning of hypertrophy of the crop. The lipoid droplets appear in considerable number in the superficial cells, and seldom fuse into one large globule, as is often the case in the mammary gland. It is of interest to note that after considerable fat has formed in the "nutritive" cells they rarely divide. Furthermore, the enormous deposition of fat in the superficial layer of cells in the hypertrophied crop over that found in the superficial cells of the non-hypertrophied crop points to the assumption that the deposition of fat in these cells is associated with cell degeneration. However, the mitochondria may appear normal in cells which contain numerous fat vacuoles. This is of interest in that it is believed by some authors that the first signs of degeneration of a cell are indicated by an abnormal form of the mitochondria. It is true that the mitochondria are usually fragmented in later stages when the cells are desquamated into the lumen of the crop.

The second and final process of pigeon-"milk" formation cannot be considered a secretory one. It simply involves a desquamation of the epithelial cells that are laden with fat into the lumen of the crop. Here we have the whole cell protoplasm plus its storage granules (fat) forming the final products of pigeon "milk." It is this

process that has been confused in certain textbooks as constituting a secretory one. This process seems unique in that a complete sacrifice of the body cells takes place to form the food for the young progeny.

The formation of pigeon "milk" seems to differ from the sebaceous "secretion" in that the entire cell does not become vacuolated and disintegrated until it has become desquamated into the lumen. According to Dahlgren and Kepner (1925), the structure of the oil "glands" of birds is similar to the "lactating" pigeon crop. Here is found a definite stratified epithelium which proliferates from the basal layer of cells and takes on a definite characteristic shape during hypertrophy like that found in the lobes of the pigeon's crop. Distinct vacuoles containing oil are observed to appear in the vicinity of the nucleus. The chief difference in the mechanics of the formation of the final products of the oil glands of birds and that of the pigeon's crop seems to lie in the fact that the outermost cells in the oil glands of birds appear to break down completely by vacuolization and subsequently to empty their contents into ducts, while in the pigeon crop the cells are sloughed off *in toto* into the lumen, and subsequently disintegrate.

Recently Slegg (1926), working on the anal glands of ground squirrels, reported a process in the formation of their specific "secretion" that is somewhat similar to that of pigeon "milk." The promised detailed histological account of the formation of the "secretion" of these "glands" has, to our knowledge, not appeared.

According to Schafer (1898), chemical studies by Hunter, Hasse, Teichmann, and others on the chemistry of pigeon "milk" have shown the usual fats, proteins, and salts but reveal an absence of any trace of sugar. Claude Bernard was one of the first to study the chemistry of pigeon "milk" and to compare it to that of milk. He found the two unlike.

The factors responsible for the formation of pigeon "milk" have not been studied to any great extent. The close correlation between the formation of the "milk" and the incubation stages of the reproductive cycle suggests that the gonads supply the factor or one of the factors responsible for crop hypertrophy. The only evidence—and that, indirect—that the gonads may play such a rôle is found

in the researches of Champy and Colle (1919). These authors described a decrease in the size of the testis which began with the hypertrophy of the crop and reached a maximum at about the middle of the incubation period. The retrogression of the testis is characterized by the absence of spermatogenesis and decrease in the number of seminiferous tubules. More than one-half of the tubules are resorbed. This process is not what normally occurs in the winter, when interstitial tissue develops. Interstitial tissue does not increase in amount during incubation and hypertrophy of the crop. Spermatogenesis is re-established 5 to 6 days after the young have hatched.

A somewhat similar condition in the gonad of the female is described by these authors. During incubation there is a marked atresia of the oöcytes of large size. This process reaches its height about the middle of the incubation period. Oöcyte atresia also takes place during the winter but never to the marked degree characteristic of incubation period.

The close correlation between these two phenomena suggest the gonad as a causative factor responsible for pigeon-"milk" formation. However, it would be premature without more direct evidence to strongly urge this view. The experimentally proved interrelationship existing between the various endocrine glands of mammals presents many other possibilities.

The psychic factor is also one which must be considered because of its evident importance in avian reproduction. Whitman, Harper, and others have shown that in the female pigeon such a factor is easily demonstrable; ovulation and subsequent reproductive activities are induced in the female without coitus or even the presence of the opposite sex. The psychic factor may play an important part by stimulating the formation of humoral substances, which may in turn effect crop hypertrophy. Since the formation of pigeon "milk" is a characteristic part of reproduction, and occurs in both sexes, it seems probable that study of the causation of the process will contribute knowledge to the general problem of avian reproduction.

The experimental work was done in the Laboratory of Zoölogy, University of Wisconsin. It is, indeed, a pleasure to record here our grateful appreciation to Professor M. F. Guyer, who suggested the problem to us and who has encouraged us throughout its develop-

ment. Likewise, we are especially indebted to Professor F. L. Hisaw for many helpful suggestions given during the course of the work. Lastly, we wish to thank Professor H. E. Jordan, of the University of Virginia, for kindly reading the manuscript.

CONCLUSIONS

1. Pigeon "milk" appears as a white slimy substance that is composed microscopically of desquamated cells laden with fat and fragments of cellular substance. The pigeon "milk" is formed in the crop of the parent birds and is regurgitated to the beak of the young squab, where it becomes the food material for the immature bird. Pigeon "milk" is not formed by the crop of the squab.

2. Desquamation of the cellular material (pigeon "milk") takes place in the crop of both male and female parent about the time the eggs are hatched. The process of pigeon-"milk" formation in male and female birds appears to be the same.

3. Histologically, the structure of the lobes of the pigeon's crop consists of stratified squamous epithelium. For convenience the epithelium may be divided into two fairly distinct layers: the superficial or "nutritive" layer, and the cells in the neighborhood of the basal membrane designated by Litwer the "proliferating" layer. Subjacent to the "proliferating" epithelium occurs a tunica propria containing blood vessels. Below the tunica propria is found a muscle layer of outer longitudinal and inner transverse fibers.

4. The hypertrophy of the lobes of the crop begins about the eighth day of incubation and continues by division of cells in the "proliferating" epithelium. The small folds of the crop are greatly enlarged and subsequently fuse to form a continuous mass of epithelium. A marked increase in the blood supply of the crop accompanies the hypertrophy of the crop epithelium.

5. At about the time the young are hatched, the outermost cells of the lobes of the crop are desquamated into the lumen, constituting the pigeon "milk." This process is continued several days while the young are nestlings. The speed of crop regression varies markedly in different birds and is probably influenced by several factors.

6. During the later stages of incubation fat globules are formed in the outer epithelial layer of cells of the lobes of the crop. The for-

mation of the fat globules is probably in the nature of a true secretion somewhat similar to that of the formation of fat globules in the epithelial cells of the mammary gland.

7. Examination of pigeon "milk" in the lumen of the crop of the parent-birds discloses epithelial cells of varying degrees of degeneration. A similar substance with further signs of cellular degeneration is found in the crop of the young squabs.

8. There is a close correlation between the hypertrophy of the lobes of the crop and the reproductive cycle.

9. Our studies confirm the view that the formation of pigeon "milk" is not a true glandular secretion but a process of desquamation of epithelial cells which contain considerable amounts of fat. The "lactating" lobes of the crop might be classified in certain respects as cytogenetic "glands."

10. Certain problems concerning the causative factors involved in the formation of pigeon "milk" are discussed.

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THE SURVIVAL OF RATS AFTER TOTAL AND PARTIAL ADRENALECTOMY AND ADRENAL TRANSPLANTATION

(Two plates)

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INTRODUCTION

A SEARCH through the literature for evidence of the rat's ability to survive double adrenalectomy (see Jaffe, 1926, for references) shows that there is a wide divergence of opinion on this point. Some of the earlier investigators found that the removal of both adrenals at one operation invariably proved fatal; others that only 10 per cent survived more than 20 hours; still others that approximately 50 per cent survived; finally a standard textbook of physiology, the 1930 edition of Starling's *Human Physiology* states (p. 952): "It is easy to keep rats alive after total extirpation of these [adrenal] organs." The present, generally accepted view is that approximately 50 per cent survive. This apparent ability of the rat to survive double adrenalectomy has been ascribed to the presence of accessory cortical tissue (Scott, 1923).

Jaffe (1926), however, in a recent study of 90 adrenalectomized rats, found that 35 per cent died within 30 days after the operation, 46 per cent lived for several months, and only 19 per cent showed no demonstrable clinical effects whatever. The point which he emphasizes is that in his opinion the individual rat must be observed over a longer period—nearly a year—before one can say definitely that extirpation of both adrenals has had no effect. In his last group of apparently normal adrenalectomized rats Jaffe found at autopsy in each instance "an accessory suprarenal mass, at or near the suprarenal sites, consisting entirely of cortical tissue." Although he frequently found microscopic accessories in normal rats, yet 80 per cent of his adrenalectomized rats died within the year. This inabil-

ity of the accessories to maintain life he attributes to their failure to hypertrophy and their subsequent exhaustion. In the 20 per cent that survived Jaffe noted "marked and rapid hypertrophy" of what he assumed to be accessories "immediately after the operation." According to these statements, therefore, accessory cortical tissue in itself would appear to be of relatively little value unless it hypertrophies.

Transplantations of the adrenal into nearly every kind of organ and tissue have met with so little success (see Oldberg, 1929, for literature) that Oldberg has been led to say, "We also believe that the adrenal cannot be auto-transplanted so that its functions are maintained."

In a preliminary report (Pencharz, Olmsted, and Giragossintz, 1930) it was stated that we had found evidence that the rat does not differ from other mammals in its inability to survive double adrenalectomy, and that transplants into the ovary have been successful. Since that announcement we have added to our data, and in this article we present the evidence in full.

METHOD AND RESULTS

The experimental animals were piebald rats, descendants of a cross between an albino and a wild rat made some fifteen years ago. The stock has been bred for generations in the zoölogical laboratory of the University of California. Only healthy animals were used—this point is especially stressed by MacKay and MacKay (1929) in their work on adrenalectomized rats. The rats were kept two in a cage (for type of cage see Long, 1925) in a room which varied in temperature between 70° and 75° F., and the animals were fed on an adequate diet which supplied all the necessary food factors. Three of the animals were 3 months old at the time of the operation, 4 animals were 7 months old, and the remainder were 4 to a little over 5 months old.

In all, 95 animals were operated on; in 62 rats both glands were removed at one operation; in 21 one adrenal was removed entirely and a variable portion of the second was excised. In 8 of the incompletely adrenalectomized animals, the fragment left behind was re-

moved some time following the initial operation. The latter two operations served a threefold purpose. In the first place they were a check on the effects of the operative procedure; secondly, we were interested in determining how far it was possible to reduce the total adrenal tissue and the animal still be able to maintain itself and continue in good health; finally if accessory tissue was present at some place other than on the incompletely adrenalectomized side, removal of the fragment should have no effect on the survival period of the animals. In 12 rats both adrenals were entirely removed at one operation, and a piece of one was transplanted to the ovary.

OPERATIVE PROCEDURE

Aseptic precautions were observed, and all of the operations were carried out by one of us in order that similar technique should be employed in each case. After being without food for 24 hours, the animal was anesthetized with ether; and first one, then the other, adrenal was exposed through the dorsal approach. When one gland was exposed, the blood vessels to it were pinched by means of a pair of fine forceps as far away from the pedicle of the gland as possible. Because of the rapid coagulation of the blood of the rat, a few seconds of such pinching were sufficient to assure a firm clot in the crushed vessels, so that, when the gland was removed a few moments later, there was practically no hemorrhage. In each case the gland was completely removed with the bit of fat immediately surrounding it, special precautions being taken to avoid crushing the gland. If the gland is crushed or removed piecemeal, it is impossible to be certain that minute fragments may not have been left behind to undergo hypertrophy and later be confused with accessory tissue. Furthermore, it is practically impossible to shell the gland out of the capsule without leaving some cortical tissue behind. The only safe procedure is to remove the gland whole and preserve it for sectioning. Even with the aid of a binocular dissecting microscope, minute fragments may readily escape detection; and if there is any question of the completeness of the operation, examination of serial sections of the glands is the only reliable check. This procedure was followed in all cases surviving more than 14 days.

HISTOLOGICAL TECHNIQUE

All excised tissues were preserved in fixatives and stored in alcohol. For fixing, Bouin's, Benda's, and Helly's fluids were employed. When it was desired to check the completeness of the initial operation, the original glands were cut 10μ thick, whereas tissues removed at autopsy or necropsy were cut 8μ. Eosin and Delafield's haematoxylin were the stains used, and Benda's fixed material was cleared in xylol and mounted in balsam.

POSTOPERATIVE OBSERVATION OF ANIMALS

Each animal was examined twice daily, and body weights of totally adrenalectomized rats were taken each morning. Partially adrenalectomized rats and rats with transplants were weighed each morning for the 2 weeks immediately following the operation. If they then showed no loss in weight and appeared to be in good health, body-weight determinations were subsequently taken once a week throughout the remaining period of observation. In a number of cases anal temperatures were also recorded daily.

TOTAL ADRENALECTOMY

This operation, although followed by a certain degree of anorexia, is apparently not so severe as to interfere seriously with the animal's normal behavior for a few days immediately following adrenalectomy, as is shown in the following case.

Rat ♀ *BH 3200.*—Both glands were removed at 2:00 P.M. Vaginal smear examination revealed a late pro-oestrous smear, and she was placed with a normal male. A vaginal smear examination on the following morning showed the presence of spermatozoa and plug. This animal died 11 days after the operation. At autopsy embryos were found *in utero.*

All the animals in this group showed the classical symptoms of acute adrenal insufficiency, viz., progressive loss in weight, myosthenia, anorexia, epistaxis, intestinal hemorrhage, and in a number of cases convulsions just before death. The chief characteristics of acute adrenal insufficiency, however, are progressive loss in weight and a sudden drop in body temperature. Table I gives the survival periods and change in weight of these animals. The following is a more detailed account of a typical case.

Rat ♂ W 6126.—Weight before operation, 200 grams. Body temperature, 38° C. Body weights for the 6 days immediately following the operation are as follows: 193, 190, 185, 173, and 162 grams. The average anal temperature for the 5 days immediately following the operation was 36.9° C. Intestinal hemorrhage was observed on the fourth day (blood adhering to bulb of thermometer);

TABLE I

SURVIVAL PERIODS OF 62 RATS FOLLOWING COMPLETE ADRENALECTOMY

No. of Rat	Body Weight (Grams)		Survival Period (Days)	No. of Rat	Body Weight (Grams)		Survival Period (Days)
	At Operation	At Death			At Operation	At Death	
♂ W 2392....	225	190	2	♀ GH 2136....	136	111	8
♀ GH 3134....	173	154	2	♂ GH 2701....	139	128	8
♂ BH 2390....	248	209	3	♂ W 3602....	206	156	8
♀ BH 2113....	130	103	3	♂ W 3703....	120	109	8
♀ GH 2412....	172	146	3	♂ B 2225....	256	194	9
♀ GH 3700....	110	105	3	♂ W 2541....	70	70	9
♀ BH 1380....	290	245	4	♂ W 2700....	140	112	9
♀ GH 2112....	125	114	4	♀ W 4101....	122	104	9
♂ W 4000....	343	300	4	♀ BH 4904....	168	138	9
♀ GH 4207....	112	108	4	♀ W 5000....	170	143	9
♀ W 5202....	115	101	4	♂ B 2117....	175	155	10
♀ BH 2316....	178	158	5	♂ BH 2215....	210	164	10
♂ W 3001....	150	142	5	♂ W 1653....	176	138	11
♀ GH 3101....	168	150	5	♀ W 1912....	120	100	11
♂ W 3702....	114	98	5	♂ W 3000....	158	150	11
♀ W 1317....	172	160	5	♀ BH 3200....	136	112	11
♀ B 1554....	157	138	5	♀ W 3202....	166	124	11
♂ W 1230....	236	194	6	♀ W 3901....	172	140	11
♀ W 1916....	162	144	6	♀ W 1914....	145	129	12
♀ W 1120....	200	174	6	♀ BH 2119....	190	145	12
♂ W 6126....	200	160	7	♂ W 3201....	178	154	12
♀ BH 6127....	193	156	7	♀ W 3600....	150	115	12
♀ BH 1454....	182	140	7	♀ W 1319....	196	167	12
♂ W 2004....	242	228	7	♀ B 1754....	207	164	13
♂ BH 2311....	192	150	7	♀ W 4102....	103	90	13
♀ GH 3102....	130	116	7	♀ W 4026....	125	102	13
♂ W 3332....	190	136	7	♀ BH 4254....	180	148	14
♀ W 1121....	218	187	7	♀ BH 1477....	235	180	15
♀ BH 4454....	160	148	7	♂ W 4026....	125	102	15
♂ BH 1032....	172	143	8	♂ W 1229....	296	256	16
♂ W 2035....	358	304	8	♀ W 2803....	156	124	18

on the fifth day its anal temperature dropped below the range of the clinical thermometer to approximately 30° C., and its body weight was 160 grams. On the following morning the rat was found dead in its cage. Autopsy revealed complete depletion of the abdominal fat; the upper portion of the small intestine was filled with blood; some blood was also found in the pyloric end of the stomach.

Examination of Table I will show that not a single one of this group of 62 rats survived more than 18 days, and more (9 animals) died between 7 and 8 days than at any other period. The average loss in weight was approximately 17 per cent of the pre-operative weight, with little variation from group to group. It was thought that in the case of the two rats which lived longer than the others,

TABLE II

In the animals listed below, one adrenal was entirely removed, only a small portion of the remaining adrenal being left *in situ*. All of these rats were indistinguishable from normal animals.

No. of Rat	Body Weight (Grams)		Interval between Operation and Necropsy (Days)	Remarks
	At Operation	At Necropsy		
W 1611 ♂	150	311	181	Right complete Left incomplete
GH 4126 ♀	167	207	138	Left complete Right incomplete
B 2804 ♀	160	210	87	Right complete Left incomplete
B 2611 ♀	140	164	47	Left complete Right incomplete
W 4103 ♂	107	151	38	Left complete Right incomplete
W 3100 ♀	112	146	34	Right complete Left incomplete
W 2025 ♂	258	272	Died of hemorrhage in attempt to remove fragment	Right complete Left incomplete

viz., 16 and 18 days, there might possibly have been incomplete removal of both adrenals; but histological examination of the glands showed that the removal had been complete. Careful search of the general region of the adrenal sites failed to reveal the presence of macroscopic accessory adrenal tissue.

It is evident that if any of these 62 rats had microscopic accessory tissue it was incapable of undergoing hypertrophy sufficiently to prolong the animal's existence.

PARTIAL ADRENALECTOMY

In this operation the right adrenal was, as a rule, removed first, and then the left was exposed. Eyelid retractors were used to hold open the wound, the viscera being pushed back with sterile cotton. With right-angled iridectomy scissors, the greater part of the adrenal was now cut away, only a small fragment being left attached

TABLE III

SUMMARIES OF INCOMPLETE ADRENALECTOMIES

In the animals listed below, one adrenal was entirely removed, only a small portion of the remaining adrenal being left *in situ*. Some time following this operation the piece left behind was removed.

No. of Rat	Body Weight (Grams)		Interval between Operations (Days)	Body Weight at Death (Grams)	Survival Period (Days)
	First Operation	Second Operation			
Right Adrenal Entirely Removed; Portion of Left Adrenal Left Intact					
W 4516 ♀	168	194	45	171	2+
W 8340 ♀	182	223	104	193	5+
W 3805 ♀	154	160	89	137	9+
GH 5127 ♀	158	210	118	183	11+
Left Adrenal Entirely Removed; Portion of Right Adrenal Left Intact					
W 5100 ♀	162	170	22	143	5+
W 3957 ♀	200	220	82	182	7+
BH 3330 ♀	130	152	97	121	8+
W 3502 ♀	170	238	96	197	11+

to the blood vessels. To rule out any possible difference in the right and left adrenals, several operations were performed in which a fragment of the right adrenal was left in place (Table III).

Corresponding with the varying sizes of the fragments left *in situ*, the rats can be arranged in a series on the basis of the severity of insufficiency symptoms. In those instances in which a relatively large piece of one adrenal remained (approximately 5 per cent), no marked symptoms of insufficiency could be detected. All of these rats gained in weight (see Tables II and III) and ate normally. One rat (BH 3330, Table III) mated and passed through normal pregnancy, and gave birth to 11 young 90 days following partial ad-

renalectomy. If, however, the remaining portion of the gland was removed later (Table III), the rat soon began to show deficiency symptoms and died within the usual survival period following total adrenalectomy. When a somewhat smaller portion of the gland was left behind, definite symptoms of insufficiency appeared, notably loss in weight or inability to gain in weight (Table IV). In spite of these symptoms the rats continued to live, and, aside from inability

TABLE IV

Similar to Table II, except smaller piece of one adrenal left behind.

No. of Rat	Body Weight (Grams)		Interval between Operation and Necropsy (Days)
	At Operation	At Necropsy	
W 2902 ♀	210	208	38
W 3601* ♀	200	200	33

* See text for detailed history of this case.

to gain weight, appeared quite normal when they were killed for necropsy.

Rat ♂ W 3601.—Right adrenal was removed completely; small portion of left gland left *in situ*. At the time of the operation, weight was 200 grams; 6 days later, 188 grams; 13 days later, 208 grams; 14 days later, 200 grams; on the eighteenth and nineteenth day following the operation, there was a slight nose bleed; otherwise no symptoms of adrenal insufficiency could be detected. The rat to all appearances was in good state of health, and was killed on the thirty-third day. Necropsy showed the visceral organs to be normal, a fair amount of abdominal fat was present, no trace of adrenal tissue on the right side; fragment on left side recovered. Figure 1, Plate I, shows a photomicrograph of that portion of the left gland which was removed at the time partial adrenalectomy was carried out; the section passes through the region of greatest injury. It will be seen that a narrow band of cortical tissue is missing from about one-third of the circumference. This shows what a small amount of tissue must have been left behind in this case. Figure 2 shows the degree of hypertrophy this fragment underwent in 33 days. That the tissue shown in this figure is the original fragment and not hypertrophied accessory cortical tissue can be readily seen by comparing the amount of injured capsule in it with what is missing in the original gland.

With still smaller fragments left *in situ* the rats were able to survive only slightly longer than after total adrenalectomy. Table V lists all such cases, and a more detailed account of one case is given below.

Rat ♀ *BH 3331.*—Right adrenal entirely removed; very small piece of left gland left behind. Pronounced insufficiency symptoms—weight dropped steadily from 180 to 141 grams in 28 days. Rat killed when evident it would survive only a day longer at the most. At necropsy, no adrenal tissue on the right side, and no macroscopic evidence of tissue on the left side. The blood vessels at left adrenal site and tissue adhering were removed nevertheless, and sectioned. The remnant of adrenal tissue proved to be about ½ mm. in thickness. Figure 3, Plate I, shows a section of the original adrenal through the region of greatest injury; Figure 4 shows the hypertrophied fragment; and Figure 5 shows a small area of Figure 4 enlarged to show that the tissue was healthy and showed no signs of exhaustion.

It is evident that in none of the rats listed in Table V in which a very small fragment of one gland was left behind was there evidence

TABLE V

In the animals listed below, the fragment left *in situ* was too small to maintain them. All of them died of acute and subacute adrenal insufficiency.

No. of Rat	Body Weight (Grams)		Survival Period (Days)
	At Operation	At Death	
W 3501 ♀	192	148	19
W 7137 ♀	174	134	20
GH 3701 ♂	110	100	10

of sufficient accessory tissue to maintain it in good health; and it is especially significant that in those cases (Table III) in which the remnant of the gland was sufficient to keep these animals alive, its subsequent removal brought on at once insufficiency symptoms and death. In these cases, also, accessory tissue, if present, did not hypertrophy sufficiently to prolong the animal's life.

ADRENAL TRANSPLANTATION

Since it is known that removal of one ovary has no effect on the subsequent health of the rat, and since the ovary is readily accessible, a check on the experiments described above was carried out in the following manner:

Both adrenals were removed in the usual manner and a fragment of cortical tissue inserted through a small slit into an ovary. Success in this operation depends on avoidance of hemorrhage; if there is

PLATE I

FIG. 1.—Section through the region of greatest injury of original adrenal. Note that the piece left behind is limited to a narrow strip of the outer cortical zone. (W 3601 ♂.) ×25.

FIG. 2.—Shows the extent to which the fragment left in place hypertrophied during an interval of 33 days after partial adrenalectomy. This animal was in good state of health throughout this period of observation. (W 3601♂.) ×25.

FIG. 3.—Section through region of maximum injury of original adrenal. Note the extremely small amount of tissue left behind. (BH 3331 ♀.) ×25.

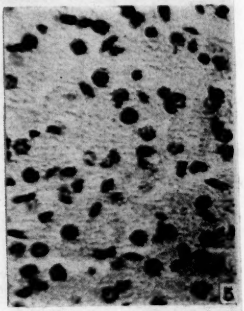
FIG. 4.—Shows the extent to which the fragment hypertrophied during an interval of 28 days following partial adrenalectomy. See text for detailed history. (BH 3331 ♀.) ×25.

FIG. 5.—A small area of Figure 4 enlarged to show that tissue was healthy. (BH 3331 ♀.) ×628.

PLATE I

3

4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bleeding, one can be certain of failure of the fragment to "take." Furthermore, the time in the oestrus cycle is of importance; transplantation proves a failure during oestrus, when there is greatest activity, and a cut into the ovarian capsule is almost invariably

TABLE VI

Summaries of operations carried out on 7 rats in an attempt to determine the possibility of adrenal transplantation. In each case one adrenal was entirely removed, only a small portion of the remaining adrenal being transplanted to the ovary. Some time subsequent to this operation the ovary containing the transplanted tissue was removed.

No. of Rat	Body Weight at Transplantation (Grams)	Second Operation		Body Weight at Death (Grams)	Survival Period (Days)
		Interval (Days)	Body Weight (Grams)		
BH 2417	200	44	195	163	8
GH 1071	158	42	186	157	15
W 1859	200	32	224	172	8
W 4360	149	27	160	138	9
BH 9320	200	24	209	148	15

TABLE VII

Operations same as in Table VI, but in these cases the transplants failed and the rats died of acute and subacute adrenal insufficiency.

No. of Rat	Body Weight at Transplantation (Grams)	Body Weight at Death (Grams)	Survival Period (Days)
BH 1755	179	137	14
BH 2901	210	150	15
B 1858	190	156	20
W 3400	168	130	21
W 1454	154	147	22
GH 1857	187	153	30
W 1980	123	107	32

attended by hemorrhage. Our successful "takes" have occurred during the "interval," i.e., when the vaginal smear shows leucocytes and epithelial cells only. It is also important to make the transplants at the cephalad end of the ovary near the fat body, rather than at the end near the oviduct. Out of 12 attempts we had 7 failures and 5 successes (see Tables VI and VII). In the failures the rats died in 14 to 32 days with all the symptoms of insufficiency. Section of the

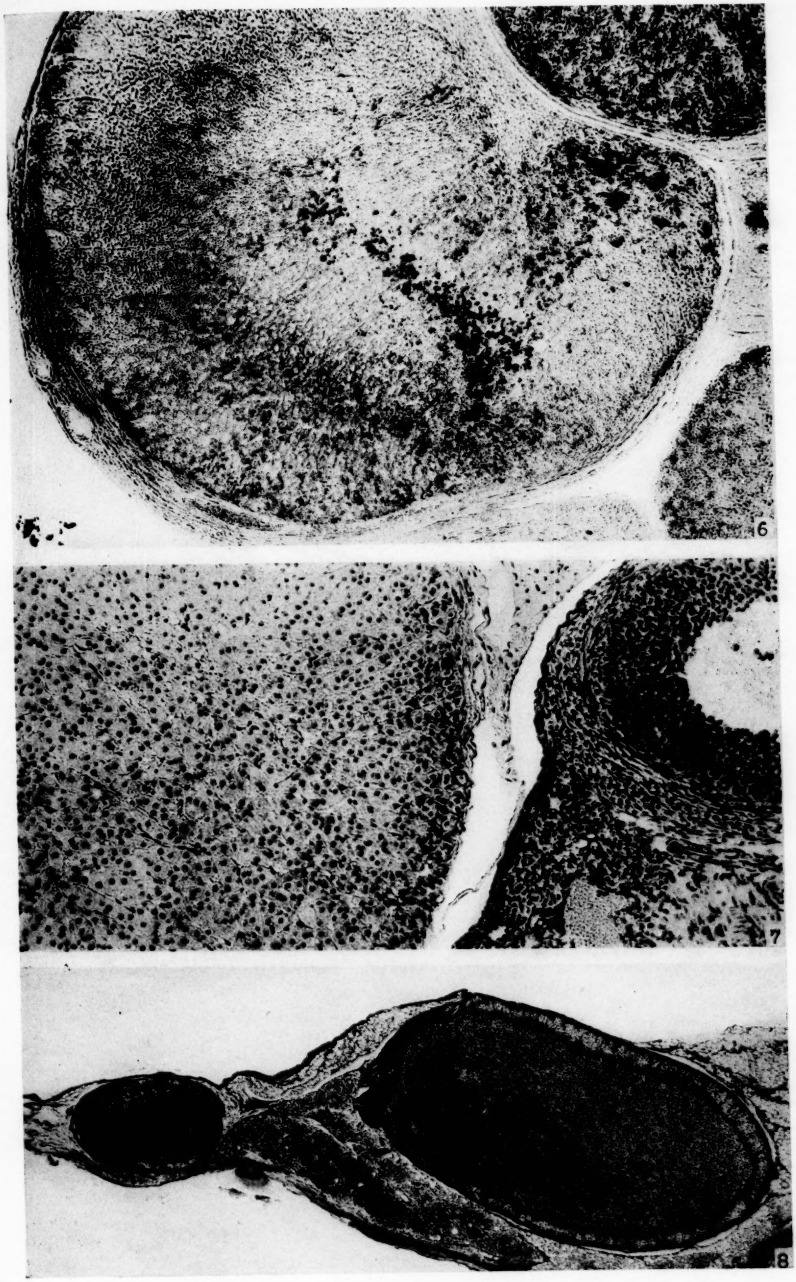
PLATE II

FIG. 6.—Adrenal transplant in ovary. Ovary fixed in Benda's fluid. Note that the adrenal capsule does not completely surround the transplanted tissue. Both adrenals were removed completely, and a small portion of one adrenal was transplanted to the ovary. This animal was kept under observation for 44 days, when the ovary was removed. It died 8 days later. (BH 2417 ♀.) ×54.

FIG. 7.—Adrenal transplant in ovary. Ovary fixed in Bouin's fluid. This animal was in excellent condition throughout the period of observation (42 days), at the end of which time the ovary was removed. It died 15 days later. Note that the transplanted tissue consists of cortical tissue only. (GH 1071 ♀.) ×107.

FIG. 8.—Normal adrenal and accessory adrenal found in rat. The normal adrenal is cut tangentially so that the medulla is not shown. The central portion of the accessory took a deeper stain, but the cells appear to be of the same character as the neighboring cortical cells. ×22½.

PLATE II



ovary after autopsy showed in 2 cases that the fragment had become necrotic. We are inclined to believe that either too small an amount of adrenal tissue was introduced into the ovary in other failures or that, while the graft was in the process of "taking," insufficiency symptoms became too severe for recovery. We are now employing the following technique, which bids fair to increase the percentage of successful "takes": one adrenal is totally removed and a fragment inserted into an ovary; not until several weeks later (usually 3) is the other adrenal removed.

In the 5 cases of successful transplantations there were no symptoms of insufficiency; weight increased in 3 cases and was maintained at a level in the other 2 cases. When these rats had lived longer than any of our totally adrenalectomized rats and were still in a healthy condition and giving every indication of living on indefinitely in health, the ovary containing the transplant was removed. Insufficiency symptoms now appeared, 2 rats died in 8 days, 1 in 9, and 2 in 15 days. Had there been accessory tissue at or near the site of the adrenals, it should have had every chance to hypertrophy, since the second operation in which the graft was removed did not involve that region at all. Figures 6 and 7, Plate II, show sections through two ovaries containing successful adrenal transplants. In each case the tissue appeared to be healthy. In Figure 6 it will be seen that the adrenal capsule extends only partly around the graft showing the original boundary of the piece inserted; and it is also to be noted that in both cases the graft consists only of cortical tissue.

ACCESSORY CORTICAL TISSUE

Descriptions of accessory tissue in the rat seem to indicate that it is present generally in microscopic amounts. Out of some 500 rats we have examined, we have found only 1 rat in which there was macroscopic evidence of an accessory. This was about the size of the head of a pin and located approximately $\frac{1}{2}$ cm. from the main adrenal on the left side. It was immediately recognized as an accessory and removed for sectioning. Unfortunately it was discovered at a necropsy for another purpose, so that it was not possible to see whether this accessory could maintain the life of the rat in absence of the main adrenal bodies. Figure 8, Plate II, shows a sec-

tion through the adrenal and accessory; it has a capsule, which, however, does not extend completely around the surface but is invaginated at one end. The main adrenal gland is cut somewhat tangentially, so that no medullary tissue shows in this section. Compare the one case of gross accessory found by Jaffe (1926, p. 455) in which an accessory was not able to maintain the existence of the rat in the absence of the main adrenal bodies.

CONCLUSIONS

1. The rat is no exception to the rule that in mammals adrenal insufficiency symptoms and death follow total adrenalectomy.

2. The rat is able to survive if only a small fragment of cortical tissue is left; less than 5 per cent of the original gland.

3. The severity of insufficiency symptoms is roughly inversely proportional to the size of the fragment of tissue left. Subsequent removal of such fragments invariably brings on severe insufficiency symptoms and death.

4. Successful adrenal transplants have been made into the ovary. Subsequent removal of the ovary causes insufficiency symptoms and death.

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[1] This paper appeared after the experiments here reported had gone to press.